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PLANT GROWTH-SUBSTANCES

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PLANT GROWTH-SUBSTANCES

THEIR CHEMISTRY AND APPLICATIONS, WITH SPECIAL REFERENCE TO SYNTHETICS

by

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Kéz kezet mos (One hand washes the other)
—*Hungarian proverb*

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Dedicated
in fellowship and with sympathy
to those who in their respective continents
work their way through college
or must study while the more fortunate relax

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NOTES

This book is based upon a series of articles which appeared in *The Manufacturing Chemist* (Messrs. Leonard Hill Limited, London) in June, July, and August, 1937 (Vol. 8, pp. 175-180, 211-216, 254-257).

References to literature not cited in full in the text or in the list at the end of the Chapter in which they are mentioned, are distinguished by a capital letter indicating the list in which they will be found. This has been done to avoid duplication, while ensuring that each list bears broadly on one subject.

The quotations ascribed to Beaumé are taken from the English translation, by John Aikin, of the second edition of Beaumé's book on Chemistry. (Warrington, 1778).

CHAPTER I

A CHAPTER FOR THE LAYMAN

> Vegetables are organised bodies, which extract from the earth the juices proper to their nature. Vegetable substances are much more compound than mineral. Their analysis is consequently more difficult: certain principles, of too great tenuity or volatility, escape us entirely.—BEAUMÉ.

THOUGHTFUL people have always been attracted by the patterned development of a plant. Practitioners of several branches of science allied to agriculture now find considerable interest in the topic of growth substances.

The subject is not really new, as its beginnings can be traced back to some observations of Charles Darwin, but it has recently become popular with remarkable rapidity. This onset of interest is possibly due less to an appreciation of the importance of the subject of growth-regulation than to a widening knowledge of the extraordinary results which can be achieved by the use of some of the growth substances lately prepared artificially. Botanists have long known that, with certain plants at least, it is possible to induce roots to grow from the middle of the stem of a plant, but that it could not be done without some trouble. It is now easy to make roots sprout on stems of almost any soft-wooded plant merely by painting them with something out of a bottle. It is not hard to understand why many laymen, as well as botanists, have had their imagination quickened.

This initiation of rooting on stems and leaves is something more than a laboratory trick, because the same substances that produce roots on leaves will cause roots to appear on cuttings more rapidly than they will appear in the ordinary way after putting into compost. To the horticulturist, therefore, the recent artificial preparations of growth-regulating substances represent a real gain of immediate practical importance. Moreover, the possibilities have not been fully explored, and it is confidently expected that the new growth-substances will be useful in grafting and in further types of propagative work in the nursery garden.

The substances are sometimes called growth-promoting substances, but that name is not sound as a general rule. It often happens that a plant that has been treated with one of these substances produces a whiskery out-growth of roots over most of its stem and a great part of its leaves, but becomes dwarfed in comparison with an untreated plant. Dr. H. L. Pearse, writing in the January, 1937, issue of the *Journal of Pomology and Horticulture*, has pointed out that the *amount* of plant-capital is not altered by the application of an artificial growth-substance such as phenyl-acetic acid, but that the *distribution* of the plant's capital is thereby altered. In other words, the more aerial root, the less stem and leaf, the sum of matter belonging to the plant being apparently unchanged. It is, therefore, better to call these substances growth-regulating substances, or, more simply, growth-substances. This book is devoted to an exposition of the chemistry of plant growth-substances; that is, substances, other than fertilizers and nutrients, that can regulate the growth of plants.

The non-chemical reader who glances at the later portions of this book and is affrighted by the array of formulae, and the words, often of more than Johnsonian ponderosity and length, with their uncouth-looking jumble of syllables hyphenated with figures and Greek letters, may think that it is something like English, but what does it mean?

It means, in brief, the story of an attempt to adapt the forces of nature whereby plants grow, not only in size, but in the increase of their several parts—roots and leaves, stems and fruit. It is the summary of an attempt to set our accumulated knowledge to a new field of endeavour: the better control of plant growth. Firstly, however, it has been necessary to understand how plants grow, and to know what substances affect their different kinds of growth.

The understanding of substances belongs to the science of chemistry, which has built up for itself that queer-looking but exact-meaning terminology which I have already mentioned. The search for answers to the questions that centre around the "how" of plant growth belongs to the science of phytology, or plant physiology, and, in what follows, I have written practically nothing under that head, because I am not a plant physiologist, and don't understand the subject sufficiently to discuss it in detail. However, some hints as to how plants grow can be gained by observing the effect of different substances on plants. The preparation and study of such substances is the job of the chemist.

The substances that can be tested on any one kind of plant fall into two groups: those which the plant can't produce for itself, and those which it can.

Plants cannot produce for themselves the mineral foods they need, and which they normally obtain from the soil; these are such things as phosphates, nitrates, silica, lime, potassium salts, and so on. They also require carbon and water, which they obtain from the air and the soil, and a plant growing from seed has a reserve of food in the seed to start life with.

Given these things, the plant grows. It gets bigger, but it also becomes differentiated, that is, it acquires parts, such as root, stem, and leaf. How does a seedling "know" that it must form a number of parts, all of them necessary, and of several different types, and how does it arrange them into the pattern which is recognizable by us, so that we can say "that is a willow, and that is a tomato plant"?

There is possibly a clue in the activities of some of the substances which the plant produces for itself. A large number of plant-products are structure-materials and reserve foodstuffs, together forming the framework of the plant and its kitchen and pantry. Other substances seem to have a directive or husbanding effect. These latter are the natural growth-regulating substances. They are also called hormones, or "chemical messengers".

Professor Boysen Jensen, on pages 115-6 of his book on growth-substances, has said: "Numerous experiments have shown that without growth-substances, growth of the shoots of higher plants cannot take place. . . . The increase in cell-wall boundaries is only one manifestation of the fundamental ability of an organism to build itself out of the materials of its environment."

As Boysen Jensen reminds us, there are several modes or kinds of growth. Growth in length or height may be due to the lengthening of a fixed number of cells, without new cells being formed: that is rather like a stretching, only, the force comes from inside, not outside. Growth in length or height may also take place by an increase in number of cells, so that the growing-point is pushed out to make more room for the older tissue. There is also a mode of growth which starts the formation of new sorts of tissue, as when a leaf-bud begins to form. This last kind of growth is due to cell-differentiation.

The effects of growth-substances that have been most intimately studied are those that arise from the first mode, namely, the increase in length of a fixed number of cells. Young seedling oats have become a favourite material for demonstrating the existence of this type of growth. This is partly because it is relatively simple to see what is happening when a certain part (the coleoptile, or first leaf-sheath) of a young seedling oat-plant that has had its tip cut off, has been treated with a trace of the substance that is being investigated. The substance is applied to one side of the cut surface of the stump (the tip, which contains the plant's own shoot-growth hormone, is thrown away). The applied substance causes the cells on the treated side to grow longer (provided, of course, that it is active as a growth-substance). The result is that the leaf-sheath bends to one side in order to relieve the strain caused by the unequal growth. The bending is easily visible.

This is the essence of the oat-test. Oat seedlings are employed because they are cheap, easily grown and handled, are responsive, and otherwise convenient.

The effect of light in causing bending of stems is known to everybody who has grown plants inside a window-space. For some years, plant physiologists sought to explain the effect of the light, before they suspected the existence of growth-substances. As soon as it became reasonably clear that there was a connexion between light and the elaboration of some growth-material by the plant, efforts were made to isolate in a pure state the substance causing the cell-elongation. Microscopic studies had shown that the observed bending was an expression of greater growth in length of some of the cells on one side of the stem.

The story of the isolation of highly-active growth-substances is an interesting one. The amount of growth-substances in the tips of seedlings was too small to offer any hope of obtaining much of it from that source. After it was noticed that urine had a marked effect in making young plant-cells become longer, efforts were made to obtain the active substance (whatever it was) from urine, which could be obtained easily in quantity. The effort was successful, and enough of a substance, at first called *auxin*, was obtained from urine, to enable its chemical structure to be ascertained. It was found that there were in urine two rather similar growth-substances; the one first discovered is now called auxin-a, the other is known as auxin-b.

In undertaking this chemical investigation of urine, the researchers did not know whether the result of their labours would after all be the finding of a substance identical with or different from the growth-hormone of oat seedlings. Even now it is not known with certainty—only with a very high degree of probability—that an auxin is in fact the natural growth-substance of the seedling oat tip. The presence of auxins in urine propounded the problem as to what they were doing there. It was found that both auxins could be isolated from some vegetable oils, and from malt, so their presence in urine can be accounted for by their being included in our vegetable foods.

Although the formulae of both auxins are known, they have not yet been prepared by purely artificial methods in the laboratory. The synthesis of the auxins is a difficult problem in chemistry.

At this point I may make a digression for the benefit of those whose ideas of chemistry are based on the reading of popular novels involving a secret chemical formula. In chemistry, a formula is not a recipe, but is an exposition of the number and kinds of atoms comprising a substance, showing also the way in which the atoms are arranged in relation to one another. Before a natural substance can be imitated in the laboratory, it is necessary to know the exact "configuration" of the molecule of the substance; knowledge of the formula, however, is far from being all that is necessary to enable even an expert chemist to make the substance on the spot. Those who are interested can find the formulæ of the auxins on pages 30 and 31.

In urine there is often a third growth-substance, the existence of which was discovered in a curious way by Fritz Kögl and others, of Utrecht. They found that it was not always possible to extract auxin-a from the mixed urines of a hospital. They therefore concentrated their attention on an "especially appreciated source"—the urine of an eighteen-year-old youth, which had been found by the oat test to be four or five times as active as ordinary urines. An isolation procedure applied to this urine led to the production of a very small quantity of crystals of a substance quite different from either of the auxins, though practically as effective in causing cell-elongation in seedling oats. The nature of this substance was a puzzle, but some hints were available, and finally the "new" substance was definitely established to be indole-acetic acid, which was already well known to chemists, but was not then known to be a growth-regulating substance. This shows that if you enter hospital, you never know what purpose you are likely to serve!

Later, indole-acetic acid was found to be present in very minute amount in yeast, from which it was laboriously isolated after more than a hundred and fifty separate treatments had been made upon three hundredweights of yeast in portions of a kilogram (about two pounds) at a time.

The story of these, and other, discoveries has been racily told by Dr. Kögl and his collaborators in the "Zeitschrift für physiologische Chemie," vol. 228, of 1934. Regarding the rôle of chance in the sequence of isolation of the auxins and of indole-acetic acid, I cannot do better than give the words (translated, of course) of Dr. Kögl and Dr. Kostermans from page 116:

> "It was a lucky accident that from urine, which contained both the growth-substances [*i.e.*, auxin, and indole-acetic acid], we isolated the "real" auxin first. If our attempts to isolate the active substance had first of all led to the finding of indole-acetic acid, probably no one would have taken the trouble to separate the active substance [auxin] existing in the residual liquors. Having acquired the knowledge that yeast and other lowly plants form a substance capable of producing cell-elongation, and identical with indole-acetic acid, one would probably have been content with that, and any variation in behaviour displayed by growth-substances in cereals [oats, especially] would have been put down to the existence of substances only incidentally present. The existence of auxins *a* and *b* would, most likely, have remained unsuspected for a considerable time."

Even now, it is not known why indole-acetic acid and the auxins behave so similarly when applied to the decapitated seedling oat and in some other tests of growth-promotion and regulation. It is usually assumed that substances that display similar physiological behaviour are at least related chemically, but between the auxins and indole-acetic acid there is no obvious similarity whatever. All three substances occur in urine because they are derived from articles of diet, but the origin of urinary indole-acetic acid is quite different from the origins of the auxins.

An important practical consequence arose from the discovery that indole-acetic acid had growth-regulating properties. Whilst the auxins were new to science, indole-acetic acid, and substances chemically similar to it, have been known for a long time. A number of ways of artificially preparing indole-acetic acid and its relatives were already known before Kögl found the acid to be a growth-substance. It did not take long to test the growth-regulating properties of it and of other substances like it. It was found that a solution of indole-acetic acid, for example, when painted on the stem of a tomato or other soft-stemmed plant caused roots to grow on the stem. From that it was but a step to the attempt to quicken root-formation on cuttings. It was found that indole-acetic acid did considerably hasten the appearance of roots on cuttings put into compost. Hence, it and its relatives are already of practical importance as an aid to horticulturists and gardeners. A number of the artificial growth-substances, including indole-acetic acid itself, are now on sale for horticultural purposes.

If you are a business man or woman, you will perhaps think that here is a field to exploit, by selling manures to which traces of the growth-substances have been added, while you claim remarkable properties of growth-promotion for "Smith's Auxonic Fertilizer". The idea has already occurred to a number of people, but I think that to translate it into practice without more knowledge of the modes of action of growth-substances would be to start at the wrong end. In my view, it is more important to investigate the old, well-tried, organic manures of the farm, and to see whether they contain the growth-substances, and in what proportions. If growth-substances are present in organic manures, a knowledge of their presence and distribution may help us to understand more about soil fertility, and possibly about the healthfulness of foods. Such research work on organic manures has not yet begun.

Some years ago, the foundation of an understanding of the rotting-down of organic materials was laid at Rothamsted, during an investigation of the decomposition of straw. One practical result was the production of the so-called artificial farmyard manure—a compost of straw. This artificial farmyard manure is not artificial in the sense that many purely inorganic fertilizers are. It derives nothing from large livestock, but is nevertheless a biological product, because many forms of living micro-organisms take part in the rotting. Artificial farmyard manure ("Adco" manure) has been shown to be the equal of farmyard manure as far as crop-producing capacity is concerned. It looks, therefore, as though the biological contribution to fertility is much the same, whether it is supplied by a few large animals or by a multitude of small forms of life.

It is too much to say that the auxins in plants, or growth-substances in general, are the very stuff of life, but the connexion between plant-growth and the natural growth-snbstances is so intimate that it is tempting to suggest that since the discovery of the modes of action of growth-substances we are a step nearer understanding the mechanism of life itself.

One paragraph more. Once Kögl had found the crystals that turned out to be indole-acetic acid, it was easy, by means of a few comparatively simple chemical tests, to say what the substance was. This was because a great deal was already known about the chemical relationships of indole-acetic acid. The chemistry of indole-acetic acid and of its relatives had been worked out over many years by researchers who were mainly interested in pure, or theoretical—as distinct from applied—chemistry; that is, many of these chemists were interested in chemistry for its own sake. They suspected little of the future importance of their work, but they were broadening knowledge, though, at the time their work was done, a good deal of it must have seemed to have no practical value except, possibly, as an exercise in chemistry. We benefit to-day from the results of the work of these people who laid the foundations of chemistry many years ago. A similar remark would be true of the work of scientists in other fields besides chemistry. That is the reason why I selected as the keynote of this book the proverb that appears on the title-page.

Legend to Fig. 1. *facing page* 40.

Root-production extraordinary. This photo shows the natural aerial roots of a tropical vine growing in the Cycad House of the Missouri Botanical Garden, St. Louis, U.S.A. In this greenhouse these roots attain a length of 40 feet, not branching at all unless they strike soil or moist material. The roots here shown owe nothing to artificial growth-substances, and the illustration is given as a matter of general interest.

According to John A. Moore (1933), from whose paper the photograph is reproduced, the vine shown had been in the greenhouse only five years. It was included by Zimmerman and Hitchcock (1935, A) in a series of plants subjected to the action of root-forming substances, towards which it behaved in no unusual way. The botanical name of this vine is *Cissus sicyoides* L. var. *Jacquini*.

CHAPTER II

ALSO FOR THE LAYMAN

How to use the Commercial Growth-Substances in Quickening the Rooting of Cuttings

> Bees burnt to ashes, and a ly made with the ashes, trimly decks a bald head, being washed with it.—[Unconfirmed quotation from N. CULPEPER'S *Herbal* (1653); *se non è vero, è molto ben trovato.*]

What to Buy.

AT present, the chief, and indeed the only commercially-available growth-substances the horticulturist and amateur gardener need trouble about, are *indole-acetic acid* and *phenyl-acetic acid.* Of these, phenyl-acetic acid is by far the cheaper; its cost puts it within the reach of all. It is, however, a less potent growth-substance than indole-acetic acid, and, therefore more of it needs to be used to be effective. Probably, too, one substance may be more useful than another for certain purposes.

These acids are slightly soluble in water (they are not corrosive, even when pure). They may, however, be obtained combined with soda, potash, or lime, in the form of salts, which are more easily soluble in water and are equally as effective as the acids themselves.

The other substances available, such as indole-butyric acid and the two naphthyl-acetic acids, and the salts of such substances, are most likely to be useful to botanists for teaching purposes, or for amateur experimentalists wishing to produce "freak" effects, such as the appearance of roots on stems.

How to Prepare

Only very low concentrations of the chosen substance are necessary. As it is difficult to make a very dilute solution straight away, the best way is to make, say, about a one per cent.

"stock" solution of the substance in water, by dissolving a known weight of the substance in a hundred times its weight of water. The dearer substances (that is, all of them except phenyl-acetic acid) are usually supplied in tubes containing one gram (28·4 grams = one ounce avoirdupois). A measure to contain a hundred grams (millilitres) of water can be bought through a druggist, but three and a half ounces of water will be sufficient approximation to a hundred millilitres.

If the acid, and not a salt, has been obtained, it will be a good plan to dissolve the acid first of all in about five or ten times its weight of alcohol or methylated spirit, which is, however, of no use for the salts. The volume of the solution is then made up with water to the final volume desired for a one per cent. solution.

The stock solution, when not required, should be stored in a cool, dark place in a bottle of dark-coloured glass, because light tends to decompose the substances.

From the stock solution a weaker solution can be made freshly as needed, by adding one part of the stock solution to as many parts of water as are desired. Dilutions of about one part of the original substance in one thousand, ten thousand, forty thousand, a hundred thousand, etc., can be made by adding one part of the stock solution to 9, 100, 400, 1,000 parts of water respectively. These final dilutions will contain respectively 100, 10, 2·5 and 1 part of substance per 100,000 of water. No alcohol or other substance except water is required to dilute the stock solution. Tap water is suitable. Any unused weak solution should not be kept, as it will probably become valueless on standing long.

A smaller measure (to contain 10 millilitres) will be found a convenience both for measuring out the stock solution and for dosing the plants.

Stock one per cent. solution can also be bought.

How to Use

In addition to solutions in water, pastes or salves consisting of the substances mixed with lanolin (wool-fat) can also be obtained or prepared. These are chiefly useful for local applications, such as smearing on definite areas of stems or wounds, and are of little practical value for inducing rooting on cuttings, which is at present the main horticultural utility of commercial growth-substances.

As the whole subject of utilization of growth-substances is new, no definite rules for their use have yet emerged, and to that extent every user must for a time be an experimenter.

The following indicates the general lines of procedure for treatment of cuttings. It may be varied to suit inclinations, and as experience is gained.

For best results, cuttings should bear some leaves.

Divide the batch of cuttings to be treated into three lots. From the stock solution, if that is of indole-acetic acid, prepare three solutions containing, say, about 5, 10 and 15 parts of acid per 100,000 of water. That is, to 10 millilitres of the stock solution add 2,000 millilitres (roughly two quarts) of water, to another 10 millilitres of the stock add 1,000 millilitres (one quart), and to another 10 millilitres add 670 millilitres of water (half a quart, and a bit). (Another way would be to get your pharmacist to make up solutions for you, reminding him that a gallon of water weighs 70,000 grains). Great accuracy is in no case essential.

Plunge the bases of one-third of the cuttings into each of the solutions in ordinary daylight at ordinary temperatures. At the end of 12 or 16 hours withdraw one-third of the cuttings from each vessel, and plant them out in potting compost, suitably labelled. At the end of 24 hours withdraw half the remainder and plant them out, and withdraw the last lot at from 36 to 72 hours after the beginning, and plant out. You will have thus nine lots of cuttings, which have been subjected to three concentrations for three periods of time. Concentration of the growth-substance and length of exposure thereto have often to be narrowly judged, but there is a certain latitude—just as in exposing a photographic negative—and at least one lot of your cuttings should have received something like the proper treatment from the suggested ranges of concentrations and times. It would add to the interest if you planted out a few cuttings treated with water only.

If phenyl-acetic acid is used, all the above-mentioned concentrations might be increased about ten times. That is, the amounts of water mentioned should be added to say 100 millilitres (3½ ounces) of stock solution, instead of to 10 millilitres.

After removal from the solutions the cuttings look just the same as before. All that has happened is that transpiration of water through the leaves has occurred, and some of the solution has been sucked up. Visible roots will not be produced before at least a couple of weeks in compost. The time will depend upon the season of the year, as well as on the kind of plant.

Experience has shown that immersion for 12 or 24 hours in a solution of about 10 parts of growth-substance per 100,000 of water is about right for several kinds of plants, and this may serve as a rough guide if you are not willing to experiment with several concentrations and times as I have suggested. Immersion in a relatively strong solution for a short time (say 10 parts per 100,000 overnight) seems to be usually better than immersion for a longer time in a weaker solution. On the other hand, an error in judgment of the concentration of a "strong" solution may lead to the preparation of a solution that is poisonous to the cuttings. Be prepared for disappointments; for death of cuttings left too long in the solutions, or in too strong a solution; and for no results, from insufficient treatments.

Some words by Richard Bradley, a famous eighteenth-century gardener, can fittingly be quoted here:

> "And again, the proportions of every Ingredient ought to be as reasonably consider'd, and not to double our Quantities over hastily, because a moderate Proportion has already begun to shew its good Effects; like some unthinking People, who believe, that the more Physick they take, the more healthful they shall be, because they have once been reliev'd from Sickness, by a small dose of it: But a few Observations to an ingenious Man, who loves a *Garden*, will soon bring him to rights in these Matters."

CHAPTER III

SYNTHESIS
(With a note on the auxins)

> Putrefaction may, I conceive, be considered as a spontaneous analysis, without heat; or a subsidence and laceration of the particles of bodies, by the weight of their mass, and by the dilatation of the fluids they contain, but aided by the external heat of the atmosphere. This spontaneous analysis disengages the aqueous, oily and saline principles of which the bodies consist.—BEAUMÉ.

IN company with many foul-smelling substances, there occur amongst the products of decomposition and putrefaction a number of ring compounds having a pleasant odour. Some of them, such as phenyl-acetic acid, have a use as perfumery synthetics, but their discovery in putrescent meat and the like was made long before any commercial application was found for them.

Indole-acetic acid has long been known as a product of natural decomposition of protein. Pathologists have devised a method of testing—the "urorosein" test—for its supposed presence in urine (see p. 85). A positive result from this test has been stated to be suggestive of intestinal disorder. There is little doubt that indole and indole-acetic acid in nature are derived from the breakdown of trytophan contained in many proteins.

It has recently been found that many ring-substituted fatty acids possess remarkable powers of altering the modes of growth of plants. The first of these compounds to be chemically identified after its growth-regulating properties had been recognized was indole-acetic acid from urine. It was originally called "heteroauxin" by the plant physiologists. Indole-acetic acid occurs in urine much more frequently than the old urorosein test indicated, and by the sensitive methods of plant physiology, it can be shown that by virtue of their content of "auxins", most urines are capable of affecting cell-growth in plants. Phenyl-acetic acid is also capable of affecting plant growth, as are a number of homologues.

Effect on Plant Growth

Looking back, it seems a little remarkable that more curiosity was not shown by agriculturists and plant physiologists about the effects on plant growth of the products of protein breakdown. From consideration of the universal esteem in which composts and animal manures have always been held, it might reasonably have been deduced that something was present in them that was not explainable in terms of N,P,K, and "humus". The fact is that elemental chemistry (with some quasi-mystical appeals to the unknown *humus*) has held this field. Investigation of the ability of certain products of protein breakdown to regulate growth in plants began without references to the origin of the substances; but when the composition of "heteroauxin" had been ascertained, that substance was found to be identical with the long-known decomposition-product, indole-acetic acid.

Already a considerable number of substances shown to possess growth-regulating properties in plants have been found to occur naturally, and many have been synthetized. A description of the chemistry alone of these substances would fill a small book, which would be out of date as soon as printed. Therefore, no attempt will be made here even to list the recorded growth-regulating substances, but an idea will be given of the synthetical preparation of some representatives of each complex group, excepting only the active phenyl compounds such as phenyl-acetic, phenyl-propionic, and *allo*-cinnamic acids. Interested chemists, having consulted the standard textbooks, will find more recent details in the substantially complete references at the end of this chapter. Being intended to introduce some physiological, as well as chemical, notions, the account is not presented in an entirely logical chemical order.

Positioning and Nomenclature

The indole molecule consists of one five-membered (pyrrole) ring (composed of one NH and four CH groups) having two CH groups in common with a benzene ring. Numbering of substituents in the pyrrole ring usually commences with the N group as 1, proceeding around the pyrrole ring away from the benzene ring. The pyrrole nucleus sometimes bears the letters α and β in place of 2 and 3, substituents attached to the nitrogen atom then having the prefix N. The practice of using Greek letters to denote position in the indole nucleus is likely to lead to confusion, especially in considering compounds, like tryptophan (3-indole-α-aminopropionic acid), in which Greek letters also serve to indicate positions in a side-chain.[1] The prefix *Pr* has also been used to indicate substitution in the pyrrole ring. The use of this prefix, which does not affect the numbering as given above, is almost extinct.

Most of the naturally occurring indole derivatives are substituted in the 3-(β) ring position. 2-substituted derivatives are perhaps less often synthetized than the 3-substituted, though 2-carboxy-3-methylindole (2-carboxy-skatole; isomeric with indole-3-acetic acid) was made by Wislicenus and Arnold as long ago as 1887. Recently, indole compounds substituted in the benzene ring have been synthetized and tested for activity upon plant growth. The prefix *bz*- is used to indicate substitution in the benzene nucleus, numbering then proceeding round the benzene ring consecutively with the numbering of the pyrrole ring. The atoms common to both rings are, as usual, not numbered.

The ring nomenclature of the indolyl and related compounds is not standardized. Sometimes the termination -ole is used, sometimes -olyl; in the case of relevant derivatives of naphthalene and other higher hydrocarbons, the tendency seems to be to retain the ring name unchanged. This confusion is unfortunate, but no greater degree of precision can be expected while the Chemical Society (Smith, 1936) write benzenesulphonic acid and phenyl-acetic acid, the simple C_6H_5 group having two names while fulfilling similar functions. The Chemical Society appear to prefer the form indole-acetic to indolylacetic, but, to the author, the latter seems desirable from the analogy of phenyl-acetic. Jackson and Manske (1935) have introduced the term indylene to indicate at least some di-substituted derivatives of indole, in analogy with phenylene.

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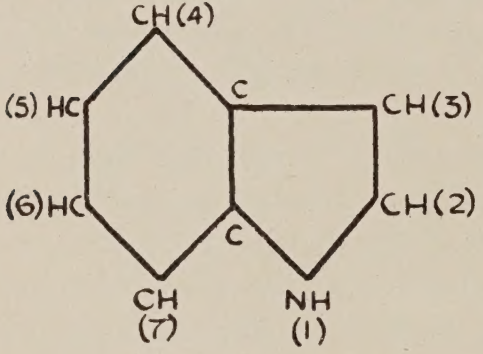
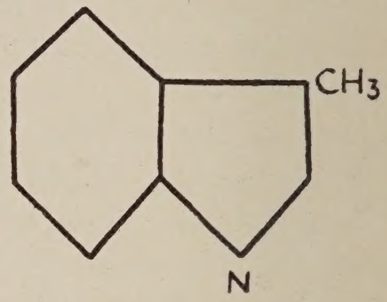
[1] The author has used the α- and β- notation in discussing the naphthalene compounds, instead of using the more modern 1- and 2-.

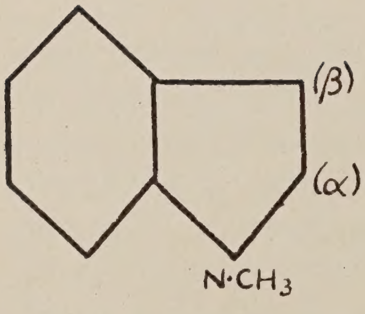
TYPICAL FORMULAE OF GROWTH-REGULATING AND ALLIED SUBSTANCES OF PHYSIOLOGICAL IMPORTANCE.



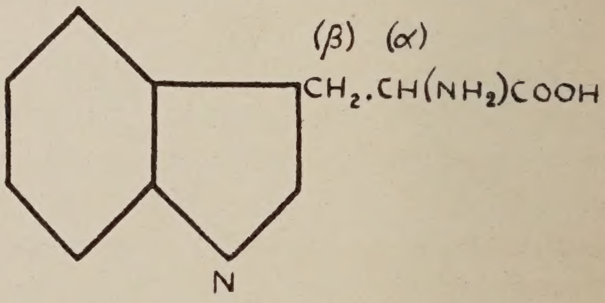
Indole;
benz-pyrrole.
$C_6H_4: C_2H_2: NH$



Skatole;
3-methyl-indole;
Pr-3-methyl-indole;
β-methyl-indole.

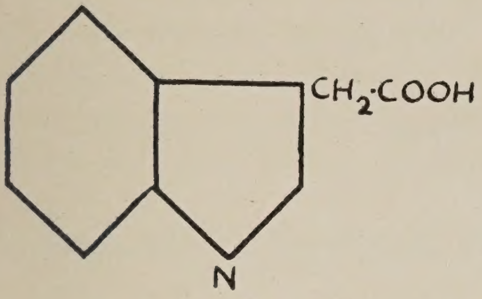


1-methyl-indole;
N-methyl-indole.

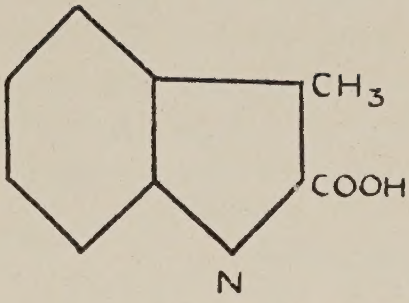


Tryptophan;
α-(3-indole)-amino-propionic acid
(optically active).

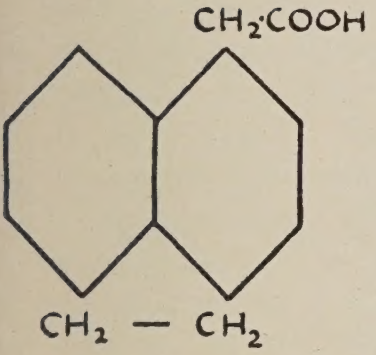
TYPICAL FORMULAE *contd.*



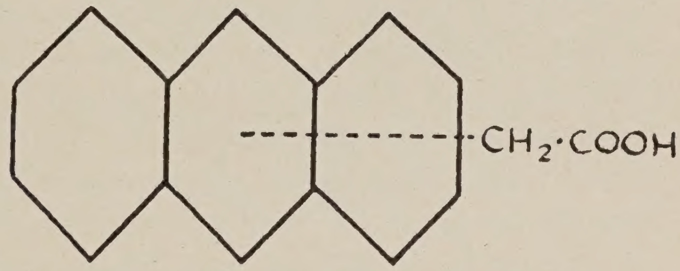
Indole-acetic acid; 3-indolylacetic acid.



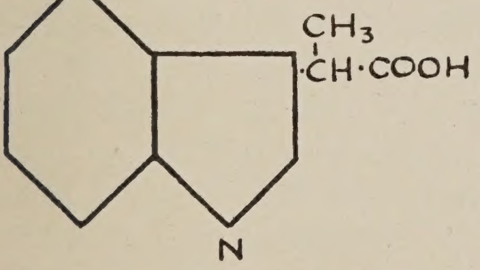
2-carboxy-skatole; 2-carboxy-3-methyl-indole.


CH₂·COOH
CH₂ — CH₂

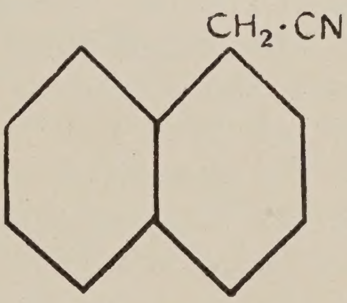
Acenaphthyl-5-acetic acid.



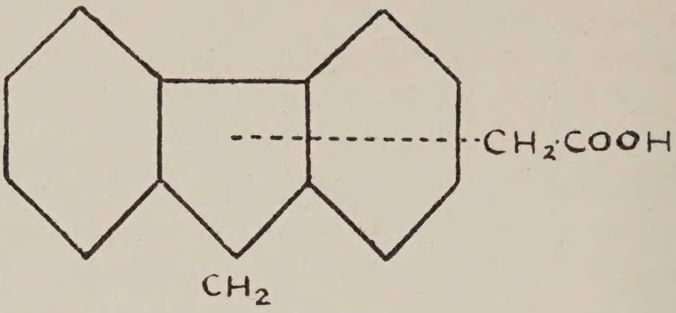
Anthraceneacetic acid.


CH₃
CH·COOH
N

(3-indolyl)-α-propionic acid. [Presumed configuration of Ellinger's (1905) indole-methylacetic acid]



α-naphthylacetonitrile.

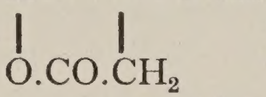


Fluoreneacetic acid.

Early Work on Indole Compounds

Indole-acetic and -propionic acids bear a close relationship to the tryptophan constituents of proteins. An excellent outline of the earlier researches on the constitution and synthesis of indole-acetic acid and its congeners is given in the article "Tryptophan," by Martha A. Whiteley, in Thorpe's Dictionary of Chemistry (1916 edition). Tryptophan is 3-indole-α-amino-propionic acid. It is somewhat surprising to learn that tryptophan, discussion of which now occupies a prominent place in many textbooks on biochemistry, was discovered so recently as 1901 (Hopkins and Cole), though the name dates from 1890. Skatole is 3-methyl-indole. A substance called skatole-acetic acid was isolated by Nencki in 1889. The synthesis of indole-3-acetic acid was not accomplished until Ellinger in 1904 performed it to follow up some problems of constitution raised by the work of Nencki and of Hopkins and Cole. Ellinger showed that it was identical with what Nencki had called skatole-carboxylic acid. In 1905 Ellinger further showed that the substance called skatole-acetic acid by Nencki was identical with indole-3-propionic acid. The preparation by Hopkins and Cole (1903) of indole-acetic acid from *l*-tryptophan by the agency of an anaerobic bacterium has now only a historical interest for chemists, but as it introduces the time factor, it retains an interest for those concerned with the biological production of growth-substances.

First Syntheses

Emil Fischer's method of ring-closure was used by Ellinger (1904) for the first synthesis of indole-3-acetic acid. The phenylhydrazone of methyl aldehydo-propionic acid, on heating with zinc chloride, or, better, by prolonged heating with alcoholic sulphuric acid (Wislicenus), yielded methyl indole-3-acetate. The aldehydo-propionic acid was prepared in fair yield by boiling aconic acid  $\begin{array}{l} HC=C.COOH \\ |\qquad\quad | \\ O.CO.CH_2 \end{array}$ with water. The aconic acid was prepared from commercial itaconic acid; for details and references, the original should be consulted. In his next paper, Ellinger (1905) described a preparation of the now usual indole-propionic (β-3-indole-propionic) acid by a tedious and probably uncommercial process, having γ-aldehydobutyric acid as intermediate. In the same paper Ellinger also described a similar preparation from aldehydo-*iso*-butyric acid of the compound isomeric in the side-chain (α-3-indole-propionic acid), for which he used the strange-sounding name indole-methylacetic acid.[1]

Relation to Animal Physiology

It is of interest that several of the more recent preparations of indole acids—some of which are now known to have remarkable effects upon the growth of plant organs—had their genesis in a study of the rôle of tryptophan in animal diets. Jackson (1929) devised a diet deficient in tryptophan, in substitution for which various indole compounds were tried. Indole-pyruvic acid was the only one of these compounds capable of replacing tryptophan in rats' diet; it caused resumption of growth in animals deprived of tryptophan. A mixture of pyruvic acid and ammonium pyruvate leads to the formation (de Jong, 1900, 1904) of the type of amino acids most common among protein derivatives—the α-amino acids; this suggests an explanation of the values of indole-pyruvic acid as a substitution for tryptophan.

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[1] The name is correct provided that the methyl group has not wandered into either ring. It suggests an interesting chemical catch. *Q.* What is methyl-acetic acid? *Ans.* Propionic acid. *Q.* Is, then, indole-methyl-acetic acid identical (for any one point of substitution in the indole nucleus) with indole-propionic acid?—Try it on your friends!

The immediate chemical interest of Jackson's 1929 paper lies in its preliminary description of new or improved methods of preparation of several indole compounds, amongst them the following: indole-3-butyric acid from application of the Fischer ring-closure to the phenylhydrazone of ethyl hydrogen α-ketopimelate (full details of this synthesis were published by Jackson and Manske (1930)); indole-3-propionic acid was prepared according to Kalb, Schweizer, and Schimpf (1926); indole-3-pyruvic acid was prepared by the method of Gränacher, Gerö, and Schelling (1924).

Berg, Rose and Marvel (1929-30), who also were interested in the animal metabolism of tryptophan, reported the preparation of indole-3-propionic acid from adipic acid. Diethyl adipate was prepared and condensed with itself (van Rysselberge, 1926) to give ethyl *cyclo*pentanone-2-carboxylate. Indole-3-propionic acid was then formed by the procedure of Kalb, Schweizer, and Schimpf (1926) as modified by Manske and Robinson (1927).

The same authors prepared indole-3-pyruvic acid by applying the Grignard reaction to indole to give indole-aldehyde (Putochin's (1926) modification of the method of Majima and Kotake (1922): the method was further modified by Berg *et al.* in that di-*n*-butyl ether was used as a solvent). The aldehyde was converted to the pyruvic acid according to the procedure of Ellinger and Matsuoka (1920).

Recent Indole-Acetic Acid Syntheses

Two syntheses of indole-3-acetic acid and one synthesis each of indylene-1: 3-diacetic acid and of two other indole acids, were reported by Jackson and Manske (1935). One of the indole-acetic acid syntheses was from diethyl-β-cyano-propionacetal $\xrightarrow{H_2O}$ acid $\xrightarrow{HCl}$ semi-aldehyde of succinic acid ($+H_2O$); this, without isolation, was condensed with phenylhydrazine; the product was subjected to the Fischer indole ring-closure, yielding the required acid. The other was a variation of that of Piccinini (1899), who used it to prepare N-methylindole-3-acetic and 2-methylindole-3-acetic acids: Jackson and Manske (1935) reported that a moderate amount of ethyl diazoacetate acting upon indole yielded mainly indole-3-acetic acid and no 2-substituted compound. "The isolation of the acid proved to be facile enough, and in some respects this synthesis is the most satisfactory yet recorded." Details of this synthesis are quoted from the original paper:

Procedure

"A dried ethereal solution of ethyl diazoacetate prepared from 50 gm. of glycine ester hydrochloride was slowly run into a Claisen flask (fitted with a long fractionating column) in which had been placed 19 gm. of indole and a trace of copper powder. The ether was rapidly distilled off as the solution was run in, so that the reaction was thus made to proceed smoothly. Finally the product was heated to 100°C. in the vacuum of a water pump, transferred to a Claisen flask, and distilled at 2 to 3 mm. There was frequently a second evolution of gas at this stage, and this was particularly pronounced in the experiment in which the proportionate quantity of diazoester was greatly increased. [This second experiment yielded less mono-acetic acid—H.N.]

The almost colourless distillate was hydrolyzed with an excess of methanolic potassium hydroxide, the solution diluted with water and the methanol distilled off. Thorough extraction of this solution with ether yielded 2 gm. of indole. The alkaline solution was then acidified with hydrochloric acid and again exhaustively extracted with ether. The combined extracts were dried with sodium sulphate and the ether distilled off. The solid, reddish residue was transferred to a suction funnel and washed with cold ether.

The indolyl-3-acetic acid readily dissolved in the ether and was recovered from the filtrate by removal of the solvent. It was recrystallized from a concentrated solution in ethyl acetate by the addition of benzene, or from hot water. The latter solvent is preferable when the acid is already of good grade. In either case, the adequately purified product melted at 167° to 168°C. (corr.), with the evolution of carbon dioxide, and this melting point was not lowered when the material was admixed with a specimen prepared from indolyl acetonitrile or one synthetized by the method to be detailed later [*i.e.*, from the cyanopropionacetal mentioned above; not otherwise reproduced here—H.N.]. The yield of indolyl-acetic acid was 12 gm. and that of the diacetic 1·2 gm."

Indylene-1: 3-diacetic acid and the other two acids described in this paper (3-methyl-indolyl-1-acetic and indolyl-3-succinic acid) are much less effective physiologically than is indole-3-acetic acid. The very effective indole-3-propionic acid can, however, be obtained by heating the 3-succinic acid. Another synthesis of indole-3-acetic acid, and syntheses of other indole compounds, were recorded by King and L'Ecuyer (1934). Several of these compounds had carboxy in the 2-position. King and L'Ecuyer prepared the first 2-acetic compound (2-carboxy-1-methyl-indole-2-acetic acid). The simple indole-2-acetic acid is not known. Still another synthesis of indole-3-acetic acid is mentioned on p. 29.

Microbiological Preparation

Biological methods of preparation are suggested by the work of Ehrlich (1912). In presence of a yeast and much sugar, tyrosine gives tyrosol (*p*-hydroxyphenyl-ethyl alcohol), and Ehrlich discovered that tryptophan similarly gave the analogous alcohol (named by him tryptophol): in absence of other sources of nitrogen, the yeast deaminized the tryptophan. In the hands of Jackson, (1929) Ehrlich's "elegant biological method" gave a yield of over 90 per cent. of crude crystalline tryptophol, but Jackson did not say whether he used the sugar or alcohol technique. Jackson (1930) has prepared tryptophol artificially by reduction of methyl (or, ethyl) indole-acetate. As tryptophol is 3-indole-ethyl alcohol, it is curious that no laboratory oxidation of it to indole-acetic acid has been recorded, though its phenyl ether has been prepared by Manske (1931). A physiological oxidation of tryptophol to indole-acetic acid has been recorded by Ward (1923).

Ehrlich's original method of preparation of indole-ethyl alcohol involved the use of tryptophan (which is expensive) and a large excess of sugar. Ethyl alcohol can be employed as source of energy for the yeast, the purification of the desired product being thereby made more easy than when sugar is used. Ehrlich (*loc. cit.*, p. 889) gave details of the preparation of indole-ethyl alcohol from tryptophan and ethyl alcohol in presence of a yeast. The requisite amount of alcohol is much smaller than the massive dose of sugar required for the sugar preparation.

It is not clear whether the ability to deaminize tryptophan in laboratory culture is peculiar to strains of yeast similar to those used by Ehrlich. The biological method, however, appears to have interesting possibilities and is probably susceptible of further refinement. It might be worth while to explore the potentialities of the microbiological deamination of casein or of its crude products of hydrolysis, the latter being obtained by either chemical or biological means. See also p. 20.

Higher Indole-Acids

Details of a synthesis of 3-indolyl-propionic acid from ω ω-diethoxy-propyl diethyl-malonate (from β-chlorpropionacetal and sodium diethyl malonic ester) are given by Davies, Atkins, and Hudson (1937). The acid prepared in this way is free from the lower homologue.

Manske and Leitch (1936) have described in considerable detail the syntheses of δ-(3-indolyl)-valeric acid and the related *bz*-alkyl-β-(5-methyl-indolyl)-propionic acid. Both these have been shown to possess the plant-physiological properties of a growth-regulating substance.

The indole-valeric acid was synthetized by the application of the Fischer ring-closure to the phenyl-hydrazone of α-keto-suberic acid and the subsequent elimination of carbon dioxide from the resulting dibasic acid (δ-3-(2-carboxy)-indolyl-valeric acid). This synthesis was strictly analogous to that of the two lower homologues (Jackson and Manske (1930); Manske and Robinson (1927)), the phenyl-hydrazone being obtained by means of the Japp-Klingemann[1] reaction from the homologous ethyl cyclo-heptanone carboxylate. This was prepared following the method first used by Kötz and Michels (1906) for the synthesis of ethyl cyclo-hexanone carboxylate. For the 1936 paper, cyclo-heptanone (suberone) was obtained by the procedure (described as "excellent" by Manske and Leitch) due to Mosettig and Burger (1930).

In outlining the preparation of the *bz*-methyl indolyl derivative, Manske and Leitch wrote that "the now well-known procedure, starting with *p*-toluidine in this case and ethyl cyclo-pentanone carboxylate, was employed".

A number of other indole derivatives, mainly of purely chemical interest at present, have been prepared by Manske (1930), including the phenyl ether of tryptophol mentioned above. The "recently much exploited" reaction of Japp and Klingemann was used in all cases to prepare the requisite phenyl-hydrazones. Manske also referred to the synthesis by Barrett, Perkin, and Robinson (1929) of 5- and 6-methoxy-β (3-indolyl)-propionic acids by methods analogous to those of Manske and Robinson (1927). Manske himself (*loc. cit.*) synthetized the 7-methoxy-3-indolyl-propionic acid and a more complex *bz*-substituted indolyl-propionate.

An isomeric form of indole has been reported by Braun and Nelles (1937), but it is not known how it or its compounds affect plant growth.

Other Compounds

For preparation and properties of indole-carboxylic acids, see the textbooks. The 3-carboxy compound is easily converted into indole.

Methyl-ketole (2-methyl-indole) and skatole (3-methyl-indole) converted into Grignard magesium halogenides, form the respective 2- or 3-methyl-1-carboxy acids when treated at room temperature with CO_2. If the CO_2 is passed in at the temperature of boiling toluene, the magnesium halogenide of 2-methyl-indole forms only 2-methyl-3-carboxy-indole. The magnesium halogenide of skatole persists in yielding the 1(N)-carboxy compound when treated with CO_2 even at 200°C., and a bath temperature of about 310° is necessary for the carboxy group to become attached in the 2-position (3-methyl-2-carboxy-indole) instead of in the 1-position.

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[1] The Japp-Klingemann (1888) reaction arises from the addition of benzene-diazonium chloride in ethyl alcohol made alkaline with KOH.

Indolyl magnesium halogenides commonly form 3-substituted alkyl derivatives from alkyl halogenides, though substitution in the 1-position may occur.

The preparation of many indole derivatives, including those mentioned in the foregoing paragraphs, and also 3-acetyl-indole (3-indolyl-methyl-ketone) and homologues, has been described by Oddo (1921). Some of his descriptions are reproduced in Houben's "Die Methoden der organischen Chemie," 2nd. edn., Leipzig, 1924; Vol. IV, p. 882.

1-methyl-indole-3-carboxylic acid, from treatment of methyl-phenyl-hydrazine-pyroracemic acid with hydrochloric acid: Hartley and Dobbie (1899).

1-methyl-indole-3-acetic acid: Piccinini (1899). See p. 23.

Few of the above-mentioned substances have been tested on plants, but they are mentioned for the sake of completeness in view of their possible synthetic or constitutional importance.

Still other synthetic compounds have been submitted to the *Avena* and other tests by Kögl and Kostermans (1935), Haagen-Smit and Went (1935 D) and others. Lists of their results are given in Boysen-Jensen's (1936 B) book, p. 52 *seq.*

The growth-regulating value of *salts* of synthetic acids has been very favourably reported by Avery *et al.* (1937), and by Zimmerman and Hitchcock (1937), whose paper on "Comparative effectiveness of acids, salts, and esters as growth substances, and methods of evaluating them" provides an interesting discussion.

Allo-cinnamic acid: see p. 69. Glycerides: see p. 66.

Naphthalene Compounds

Zimmerman and Wilcoxon (1935) prepared α- and β-naphthalene-acetic acids from the corresponding methylnaphthalenes by bromination, conversion to the nitrile, and hydrolysis of the nitrile by the procedure of Mayer and Oppenheimer (1916). It may be noted that α-naphthalene-acetic and γ-indolyl-3-butyric acids are the most powerful root-forming acids known (Zimmerman, 1936). For the synthesis of indolyl-butyric acid, see Jackson and Manske (1930). Use of a pure α-naphthyl-acetic acid free from the β-form and from naphthoic acid present in earlier preparations, was reported by Zimmerman and Hitchcock (1937). This was presumably the same as the acid prepared by Wilcoxon (1937) by a procedure based on Rousset's (1897) method. Employing the Friedel and Crafts reaction between naphthalene and ethyl chloroglyoxylate, Rousset obtained α-and β-ketonic esters in yield 50 per cent. of theory; reduction of the ketone acid then yielded the corresponding glycollic or acetic acid.

Wilcoxon's paper reviews probably all the existing methods for the preparation of naphthyl-acetic acids. It is of interest to note that Wilcoxon was unable to obtain any naphthyl acid by following the patented procedure of Wolfram *et al.* (1934).

His modification of Rousset's method gave a product (yield 47 per cent. of theory) consisting entirely of the desired α-form of ethyl naphthalene-glyoxylate.

Reduction of this with sodium amalgam gave a 40 per cent., and with hydrogen and nickel catalyst a 88 per cent., yield of α-naphthalene-glycollic acid. Reduction of the glyoxylate ester with hydriodic acid and red phosphorus gave a 90 per cent. yield of α-naphthalene-acetic acid.

The ethyl naphthalene-glyoxylate, and the naphthalene-glycollic and -acetic acids were stated to exhibit plant growth-regulating properties in varying degrees, but the details of the physiological experiments have not yet been published.

Higher Aryl Compounds

These are included in the only set of examples of the production of growth-regulating substances protected by patent (Wolfram *et al.*, 1934).

Anthracene-acetic, fluorene-acetic, and acenaphthene-5-acetic acids can be prepared by heating the hydrocarbons with chlor-acetic acid. The yields are small, but Zimmerman and Wilcoxon (1935) found that they could be improved by adding aluminium chloride to the melt. The location of the substituents in the anthracene and fluorene compounds is not known.

An Important Hint

In this series of indolyl and related syntheses it has been customary to aim at the preparation of the pure acids, often via an alkyl ester subsequently hydrolyzed. In view of work by Zimmerman, Hitchcock, and Wilcoxon (1936) it is doubtful whether the sometimes troublesome isolation of the free acid is necessary or desirable. These authors have shown that several esters belonging to the series of phenyl-acetic, naphthyl-acetic and indole-fatty acids are quite effective in initiating or otherwise affecting growth of organs and cells in plants. Methyl 3-indole-acetate indeed, was more effective in this way than was the free acid: the "natural" "hetero-auxin". The same authors state that there is no evidence at hand that hydrolysis of these esters to acids occur in plant tissues. It has, however, been shown by Zimmerman and Wilcoxon (1935) that α-naphthalene-aceto-nitrile was not only not poisonous to plants, but was very effective physiologically in the same respects as was the free acid derivable from it by hydrolysis in the laboratory.[1]

No record of a plant physiological test of indole-3-acetonitrile seems to have been made, but Jackson (1929) secured 13 gm. of the nitrile from 15·6 gm. of indole in the course of preparing indole ethylamine by the method of Majima and Hoshino (1925).

The synthesis of Majima and Hoshino represents a quite practical method of preparing indole-3-acetic acid: indole magnesium iodide, treated with chlor-aceto-nitrile in ethyl ether or in anisole solutions, yields the nitrile. The nitrile is easily hydrolyzed to the acid.

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[1] The nitrile was more effective in root-production on stems of large plants than was the free acid at the same concentration.

As some of the acids, more particularly the naphthyl-acetic acids, are prepared via the corresponding nitrile, we have an additional reason for believing that isolation of an acid is not an essential goal in the manufacture of a potent growth-regulating substance.

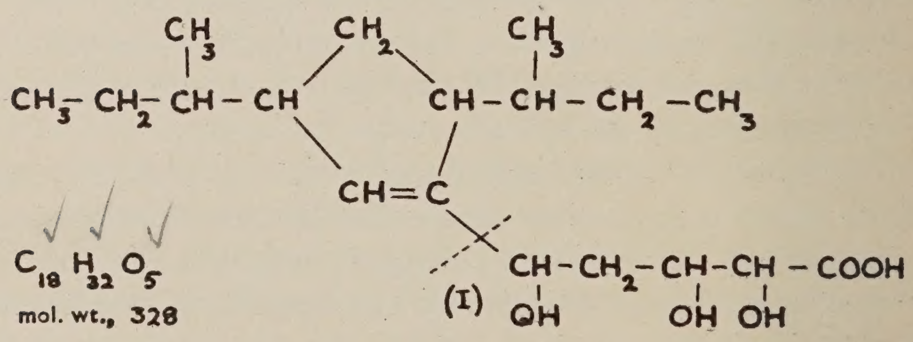
A Possible Non-biological Application

Before passing on to an account of physiological effects of these growth-regulating substances on living plant material, it may be noted that Robbins and Jackson (1937) have recorded an effect of indole-acetic acid on elongation of dead stem-wall material (cotton thread and hemp cord). These textiles stretched more, under the influence of a weight, when treated with lanolin containing 0·2 per cent. of indole-acetic acid, than when treated with lanolin alone.

The Auxins

These have not yet been synthetized. Their structure was established by the work of Kögl (1935).

The formula of auxin-a (*syn.*, auxentriolic acid) is:

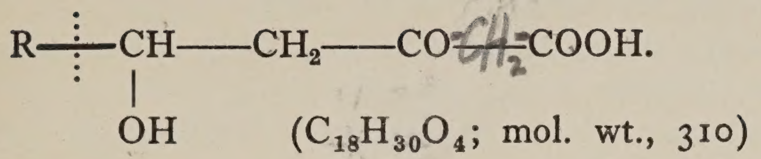


✓ ✓ ✓

$C_{18}H_{32}O_5$

mol. wt., 328

The configuration of auxin-b (auxenolonic acid) is similar, the side chain (I) being replaced by: $(R=C_{13}H_{23})$



$$R\text{—}\underset{\displaystyle OH}{\underset{|}{CH}}\text{——}CH_2\text{——}CO\text{—}CH_2\text{—}COOH.$$

($C_{18}H_{30}O_4$; mol. wt., 310)

Auxin-a is very stable towards acids, but is readily decomposed by alkalis. Auxin-b is decomposed by both acids and alkalis, whereas indole-acetic acid, though hydrolyzed by acids, is very stable towards alkalis. These properties were made use of by Kögl, Haagen-Smit, and Erxleben (1934) as showing that the growth-hormone in the tips of oat seedlings was probably auxin-a.

Auxin-a lactone, distilled with potassium hydrogen sulphate, yielded a substance probably identical with auxin-b. (Kögl, 1935).

REFERENCES A

It is believed that all modern work on the chemistry of indole-acetic acid and higher growth-substances can be obtained at first or second hand from the following lists. For earlier work see the article *Tryptophan* in Thorpe's Dictionary and the papers of Ellinger. References marked with an asterisk are of mainly botanical or physiological interest.

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CHAPTER IV

MODES OF APPLICATION

Results of Scientific Work on Effects of the Synthetic Growth-Substances Applied to Plants

THE rapidity with which the subject of synthetic growth-regulating substances has developed can be judged from the relative poverty of mentions of these substances in Boysen-Jensen's (1936) book, which contains prefaces dated June, 1936. This book is written mainly from the point of view of the academic plant physiologist, rather than from that of the horticulturist. To the chemist it will be of interest mainly on account of the extent of the lists of references to work on the physiological aspects of plant-growth stimulation; most of these, however, are of the "pre-indole-acetic era", having served to convey the ideas of botanists and plant physiologists at a time when the chemistry of plant-growth stimulants was non-existent.

Physiological Methods of Testing

The earliest methods of testing for the presence of growth-promoting substances other than fertilizers were based upon the amount of curvature occurring in a coleoptile (first leaf sheath) of an *Avena* (oat) seedling after the growing tip had been cut off and material containing the substance had been applied to one side of the cut surface. This material was often derived from another growing plant. The test depended upon the stimulation of cell-elongation, and hence of growth on the treated side, so that the cell-tissue became unequally developed, and bending was visible to the naked eye. The method was suited to the detection of very small amounts of active substance, as a relatively small number of cells were directly affected. In many cases the active substance was that absorbed from a plant-surface by a moist agar surface during a very short time, a small block of the agar being then cut out and applied to the cut surface of another (demonstration) plant, which showed by a bending response that it re-absorbed some of the substance present in the first plant. An *Avena-Einheit* (AE) has been devised. Full details of this technique and of its numerous refinements will be found in Boysen-Jensen's book.

It was soon realized that urine and the resources of biological and synthetic chemistry laboratories could provide larger amounts of growth-promoting substances than those yielded by a transverse section of a plant. The delicate oat-coleoptile (*Avena*) test has remained in use for purely physiological research, but for more practical purposes it has been succeeded by grosser tests largely based either on root-formation—often in highly unusual positions on the plant—or else on the phenomenon known as *epinasty*.[1] Epinasty is an expression by a mature plant of a set of changes due to unequal development of various parts, so that bending, and sometimes twisting, develops. For example, if one side of a stem or leaf receives more growth-promoting substance than the other, the stem or leaf will bend or curl. The phenomenon of epinasty is thus not different in principle from the simple and measurable bending of a decapitated coleoptile of an oat seedling, but visible epinastic changes in mature plants are complicated by the presence of asymmetric structures of various degrees of rigidity—such as the tapering "veins" in the leaves. For this reason epinastic changes must be regarded as gross qualitative manifestations.

Growth Promotion and Growth Regulation: a Distinction

The production of roots, or, to speak more accurately, the initiation of root-formation, is that property of growth-regulating substances of the most practical importance at the present moment. Before discussing the property of root-initiation the author desires to draw the attention of the reader to the fact that the adjective *growth-promoting* has been twice used in explaining bending tests, but that the term *growth-regulating* has been reverted to in speaking of root-production. This is because at the cut surface of a seedling coleoptile the initiation of cell-elongation is promotion of growth. On the other hand, initiation of root-formation on a mature plant, while leading to the differentiation of new tissue in some places, is often accompanied by diminished growth in other parts, and the plant may even become dwarfed (Hitchcock and Zimmerman, 1935; Pearse, 1936–7). Hence it would be illogical to apply the term "growth-promoting" indiscriminately in alluding to substances other than the nutrients in fertilizers. There seems a need to distinguish, as zoologists have done in their own sphere, between the promotion of cell growth and the initiation of tissue differentiation in plants.

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[1] Epinasty and its opposite, *hyponasty*, are together known as *nastic* movements or responses. The original papers should be consulted for fuller details, including a discussion of the hyponastic effects, epinasty being taken as the type of both for the purpose of this introductory explanation.

The adjective "growth-promoting" is sometimes applied by physiologists to those amino-acids, without which animal growth (in size) cannot take place. It is doubtful whether the adjective is valid in that connexion, which is mainly foreign to the purpose of this book. Some of the work of R. W. Jackson, and of Berg, and others, had its origin in a study of the necessity of certain amino-acids in animal nutrition.

A method of actually observing the formation of roots was reported by F. W. Went (1934), who found that the number of roots formed on cuttings of sweet pea was approximately proportional to the concentration of rhizocaline ("the root-forming substance").

Hitchcock (1935), of the Boyce Thompson Institute at Yonkers, New York State, caused initiation of roots, epinasty of leaves, and bending and swelling of stems of intact tomato, tobacco, African marigold, and other plants by making local applications of synthetic indole-acetic and -propionic acids. He made the interesting prophecy that "The fact that one homologue of 'heteroauxin' [indole-acetic acid] was found to be active in causing certain formative responses indicates that possibly other indole derivatives and perhaps other unrelated chemicals might induce one or more of these same responses." It will be seen how abundantly this remark has been justified.

Description of Methods

A description of methods may be quoted from a later paper from the Boyce Thompson Institute (Zimmerman and Wilcoxon, 1935):

> "The compounds were used as distilled water solutions or mixed with lanolin (U.S.P.).[1] In a few cases where cuttings were involved dilutions were made with Knop's solution.[2] The cuttings were placed in vials or flasks so that the basal ends were immersed in the solutions. The water solutions were introduced into the stems and petioles [the stalks of the leaves] by means of glass tubes drawn to a capillary at one end. The capacity of the tubes varied, but held on an average approximately 0·3 c.c. of solution. The capillary end of the tube was inserted into the stem or petiole and left to drain into the plant. There was considerable variation in the length of time required for the tubes to empty. Also the response of the plant varied with the rate at which the substance drained from the tubes.
>
> "Lanolin preparations were applied locally by rubbing the mixture on stems or leaves with a glass rod. . . . The usual concentration range was from 0·01 per cent. to 2 per cent."

Another technique used in later work (Hitchcock and Zimmerman, 1936) instead of injection was to dip a slit-up portion of the stem of an otherwise intact plant into a vial containing solution.

The following list of species of plants successfully used in the experiments reported by Zimmerman and Wilcoxon (1935) showed that the effects of certain growth-substances were not peculiar to a limited range of plants: African marigold, tomato, buckwheat, sweet pea, Windsor (broad) bean, sunflower, Jerusalem artichoke, dahlia, sensitive plant (*Mimosa pudica*), and goosefoot (*Chenopodium album*). A number of other unrelated plants were used in later experiments. Amongst them was the tropical vine *Cissus sicyoides* illustrated in Fig. 1.

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[1] The lanolin-smear method was introduced by Laibach (1935).

[2] Physiologically balanced and isotonic with land-plant cells, Knop's solution is a simple mixture of inorganic reagents in water.

Epinasty of leaves was induced by application of the substances in lanolin or by injecting a solution by the capillary-tube method. When the substance was applied in lanolin to a narrow zone around the stem of an active plant, leaves above and below the zone of application showed downward bending. Epinasty of any single leaf was induced by applying the lanolin paste lightly to a small region on the upper side of the petiole. There was considerable similarity in the response of plants to the cyclic compounds and to ethylene, propylene, acetylene, and carbon monoxide.

Tomato plant cuttings 5 ins. long with their basal ends immersed in Knop's solution containing the synthetic substances showed declination of the uppermost leaves within a few hours. The use of Knop's solution does not appear to be essential, as Hitchcock (1935) and others have reported a similar response to aqueous solutions of indole-propionic acid. Zimmerman and Wilcoxon (1935) did not determine the absolute lowest concentrations effective in eliciting this rapid response, but they found that the following low concentrations were effective in that way: Phenyl-acetic acid 125, α-naphthalene-acetic acid 20, and indole-butyric acid 4 parts per million of solution. On the other hand, α-naphthyl-acetonitrile did not cause an evident response in tomato plant cuttings until the second day after its application in solution to the basal ends. Whether applied thus, or injected, or in lanolin, α-naphthyl-acetonitrile evoked final results with tomato similar to those obtained with α-naphthyl-acetic acid.

Growth substances applied to one side of active shoots caused negative or positive bending, according to the concentration of the chemical, and the plant species.

Altogether eight compounds were found by Zimmerman and Wilcoxon (1935) to cause unusual activity such as nastic responses, root-initiation, etc., when applied to sizeable plants; the list has been lengthened since.

Initiation of Roots

All the compounds mentioned in the preceding section, and also anthracene-acetic, fluorene-acetic, and acenaphthene-acetic acids, caused local initiation of roots (adventitious roots) on aerial parts of growing plants of tomato, sunflower, African marigold, Jerusalem artichoke, tobacco, dahlia, and buckwheat. α-naphthyl-acetic and indole-butyric acids were especially effective in initiating roots on *stems and leaves.* The subjoined table, taken from the paper by Zimmerman and Wilcoxon (*Contrib. Boyce Thompson Institute*, 1935, 7, 211), indicates the approximate effective concentration ranges of five of the substances, expressed as percentages in lanolin. Numerical results are, of course, affected by the mode of application, the species of plant, and the type of effect sought.

Table—Effective Concentration Ranges of Five Synthetic Growth-Substances for Tomato, expressed in Percentages in Lanolin.

Chemical Substances	*Causing Negative Bending in Stem or Epinasty of Leaves.*	*Inducing Adventitious Roots.*
α-naphthalene-acetic acid	0·0100—2·0	0·1—2·0
Indole-butyric acid	0·0100—2·0	0·1—2.0
Indole-acetic acid	0·0003—2·0	0·4—2·0
Indole-propionic acid	0·0250—2·0	1·0—2·0
Phenyl-acetic acid	0·0250—3·0	1·0—3·0
Fluorene-acetic acid	0·0500—3·0	1·0—3·0

This remarkable initiation of roots is well shown by Figs. 2 and 3, taken by permission from the paper by Zimmerman, Hitchcock, and Wilcoxon (1936, p. 109). Though this paper was mainly devoted to discussing the effectiveness of esters of the cyclic acids, the mode of root-formation by the acids, esters, and the one nitrile tested was similar. Fig. 2, indeed, presents evidence of the root-initiating capacity of both α-naphthyl-acetic acid (left) and its ethyl ester (right), applied to a leaf and one side of the stem of each plant. Fig. 3 shows an especially beautiful demonstration, the plant on the left being in this case untreated, that on the right having been treated with a low concentration of methyl 3-indole-acetate only ten days before the photograph was taken. The plant shown in Fig. 3 belongs to an uncommon species—*Kalanchoe daigremontiana*—which has the property (useful for teaching purposes) of producing white roots in a dry atmosphere.

The initiation of roots on woody plants such as apple, holly, and *Cupressus* is a problem which has not yet been solved satisfactorily in all respects; there remains considerable scope for experimentation. The lanolin-smear method appears to be less generally effective with the woody plants than with such plants as tomato and *Kalanchoe.* F. W. Went (1934) and Cooper (1935) suggested that the apical ends of cuttings, and not the basal ends, should be treated with preparations of the growth substances, in accordance with the idea that "auxins" can move only downward in stems. Other workers have shown that treatment of the basal ends of cuttings with lanolin, water or dust preparations of synthetic growth substances can induce root formation.

Fig. 2.—Tomato plants, showing coloured roots grown in dry air of the greenhouse, after treatment with lanolin preparations of the growth substances. There are roots on the long stem-like leaf-stalks bending towards the camera, as well as on the true stems. The bending of the stems arose from the treatment on one side. The photographs were taken 18 days after treatment. Left: Leaf and one side of the stem above it, smeared with 0.50 per cent. α-naphthalene-acetic acid.
Right: Similar plant treated with 1.0 per cent. ethyl α-naphthalene-acetate.

Fig. 3. *Kalanchoe daigremontiana,* a tropical "greenhouse" species, here kept under reasonably dry conditions. Left: Untreated. Right: Showing the collar of white roots induced near the top of the stem ten days after local treatment with a lanolin preparation containing 0.05 per cent. methyl 3-indole-acetate.

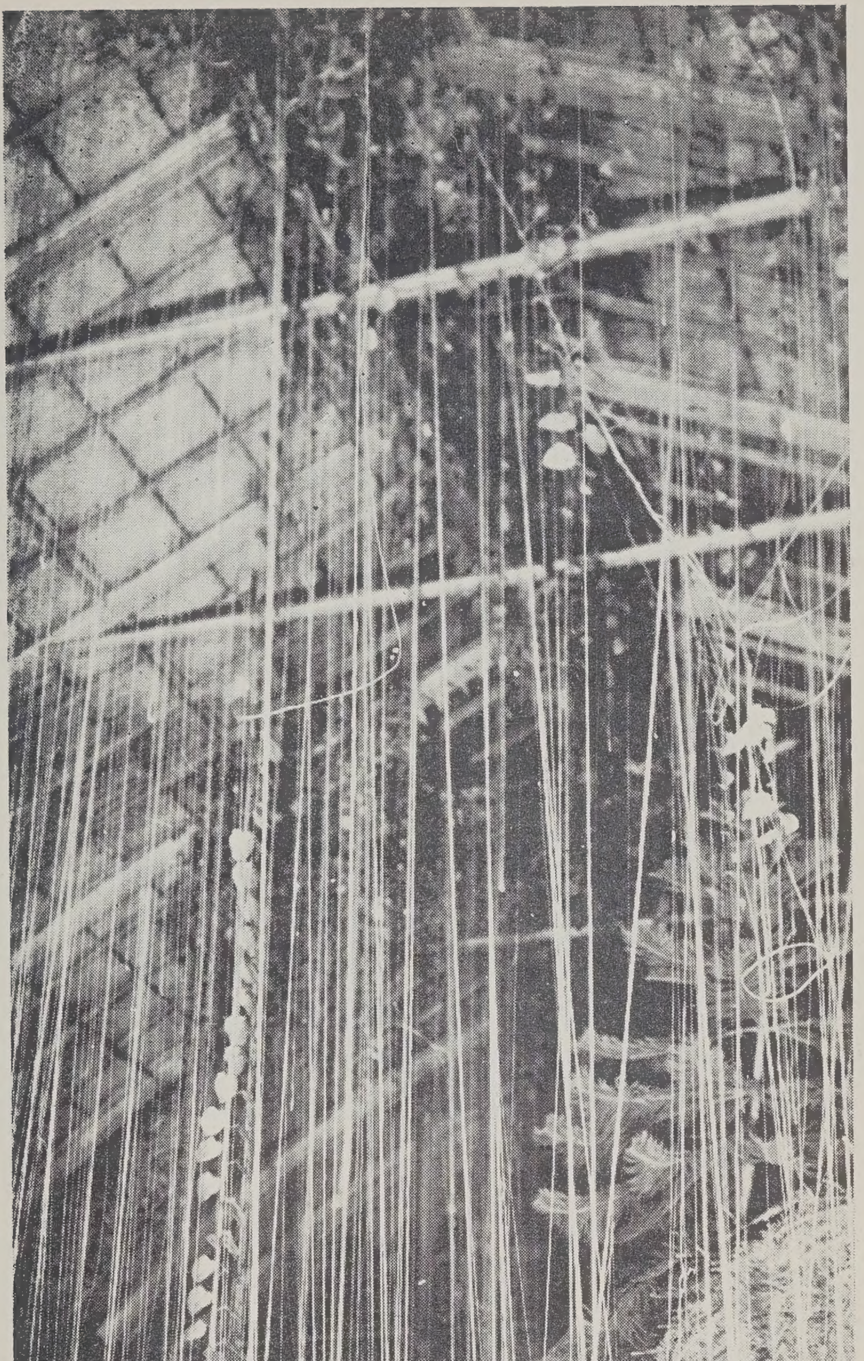

Fig. 1

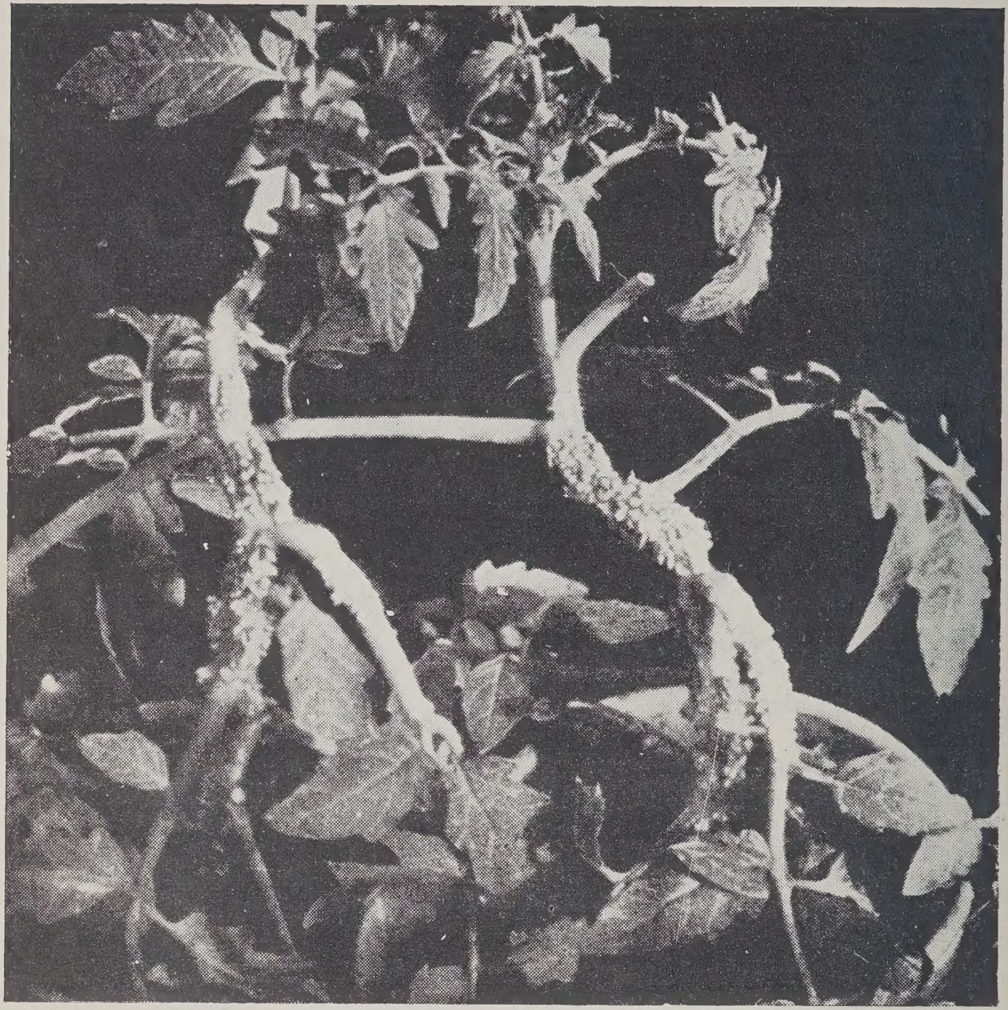

FIG. 2

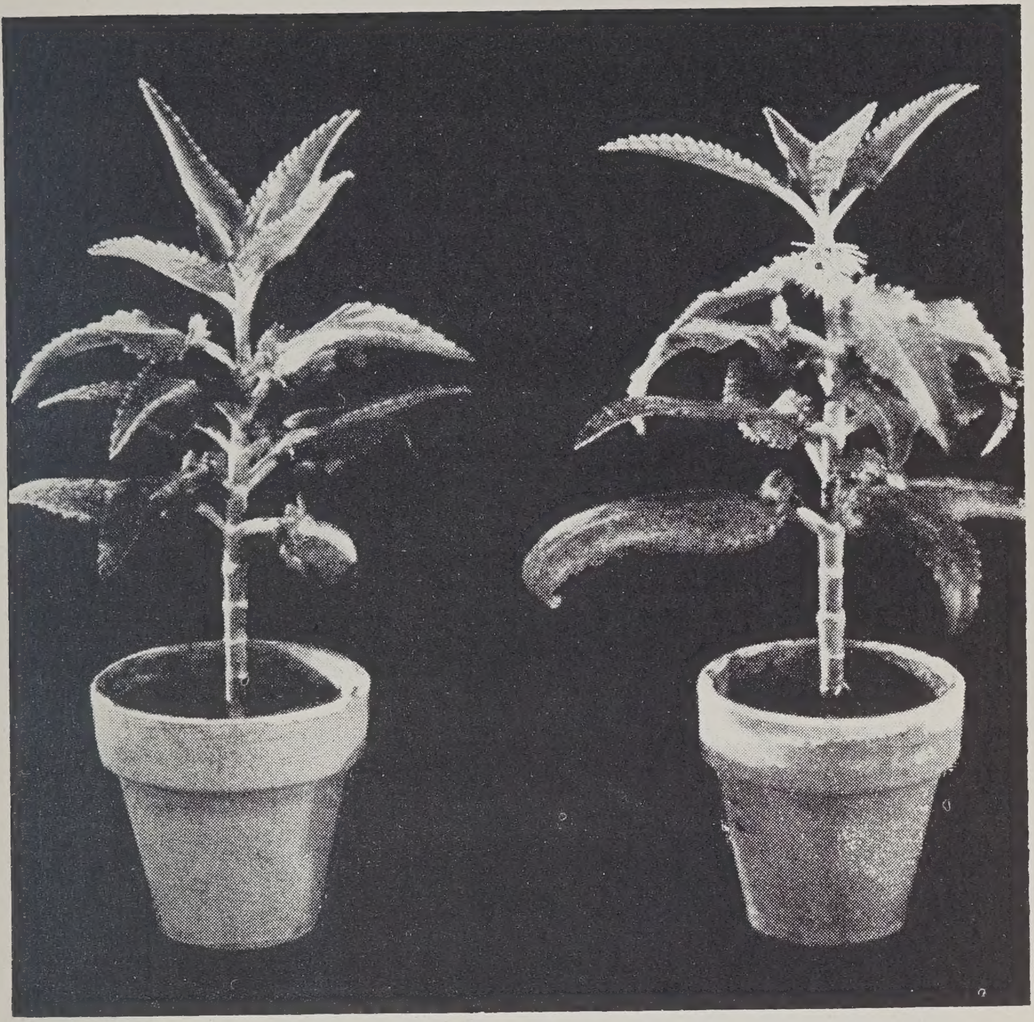

FIG. 3

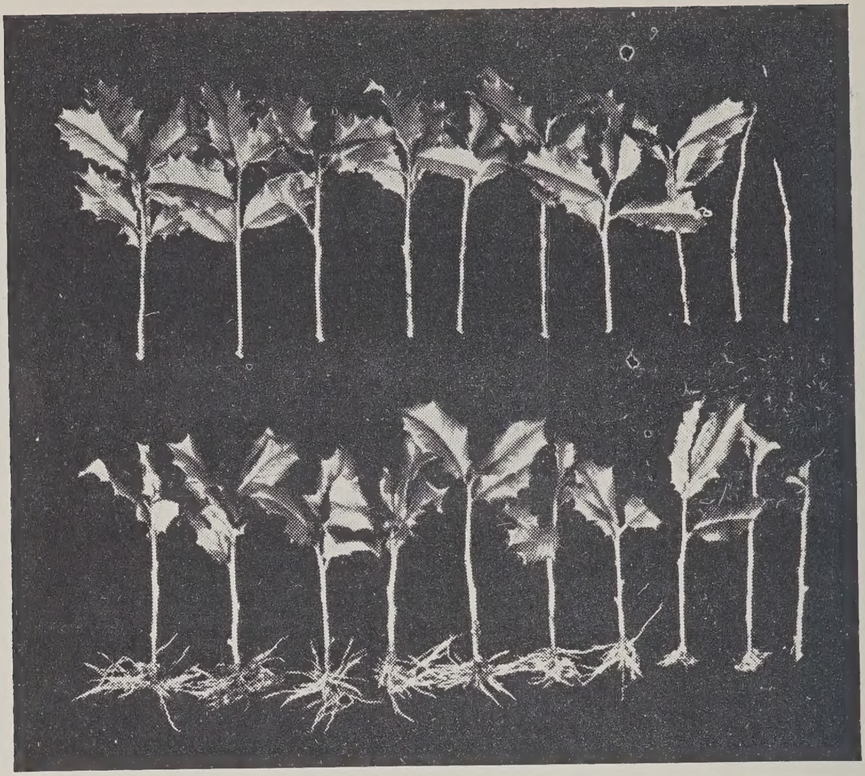

Fig. 4

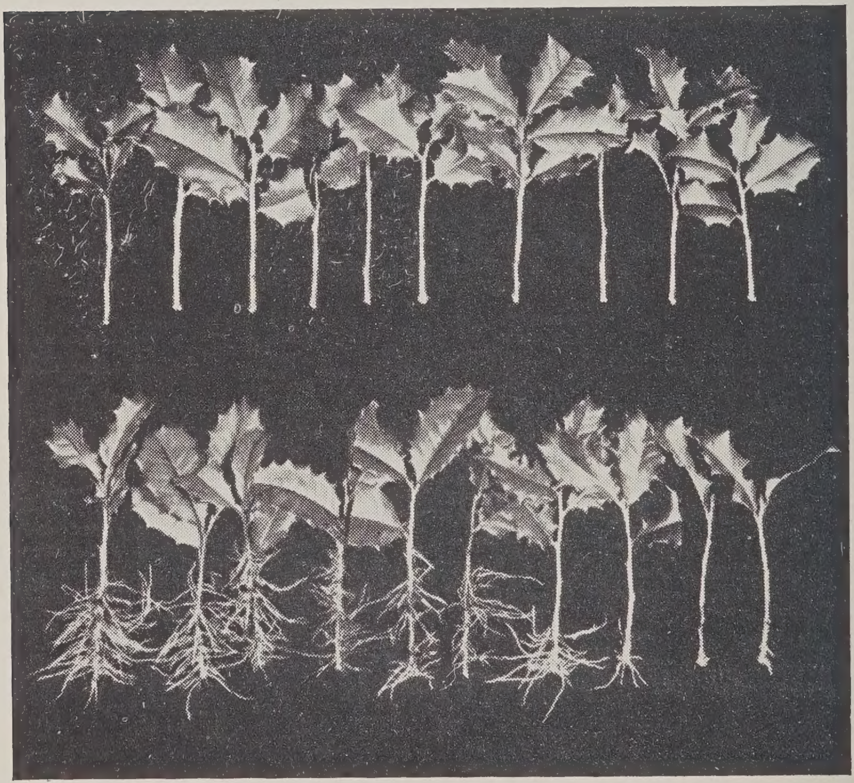

Fig. 5

Practical Methods of Using Solutions

With respect to the practical use of solutions of growth substances for inducing root-formation in woody cuttings, Hitchcock and Zimmerman (1936) recommend leaving the cut (basal) ends of cuttings for twenty-four hours or several days in aqueous solutions containing relatively low concentrations in preference to using higher concentrations for periods shorter than twenty-four hours. *Desirable concentrations of what they call the three principal substances (indole-acetic, indole-butyric, and α-naphthyl-acetic acids) would be from* 1 *to* 4 *parts of the substance in* 100,000 *parts of water.* A small quantity of alcohol may be used for preliminary solution of the substance; the naphthyl-acetic acids are all but insoluble in pure water. After treatment with the water solution for two to four days the cuttings should be placed in a potting compost. The novice should be warned not to expect to see any extensive root-formation at the end of a few days in the solution; the immediate purpose of the treatment is to enable the cuttings to absorb the growth-substance, which will then initiate root-formation while the cutting is in the compost. The formation of roots, however, usually takes place in appreciably less time than when compost alone is used.

Figs. 4 and 5 show respectively the effects of solutions of indole-acetic acid (10 parts per 100,000) and α-naphthyl-acetic acid (2 parts per 100,000) on batches of ten cuttings of American holly. These photographs are reproduced by permission from the paper by Hitchcock and Zimmerman (1936, p. 66). Similar results were obtained with

Fig. 4.—Rooting response of cuttings of American holly. The bottom row had had their lower ends dipped for 54 hours in a solution of 10 mg. of indole-acetic acid in 100 ml. of water. The top row was placed in tap water for the same time. All cuttings were then put into a compost of peat-moss and sand for five weeks before the photograph was taken.

Fig. 5.—Two sets of cuttings similar to those of Fig. 4, except that the lower row of cuttings were dipped for four days in a solution of 2 mg. of α-naphthalene-acetic acid per 100 ml. water, the controls being put into tap water for four days.

Japanese holly and *Taxus cuspidata* (Chinese yew)—also difficult plants on which to induce rooting with compost alone. It will be noticed that the cuttings that were almost leafless produced few roots, in agreement with the experience of gardeners who have used compost only. Rooting responses of a number of other species of plants are described in the paper.

Japanese maple was one of the few species of woody plants which responded consistently to treatment with lanolin preparations, concentrations of from 25 to 100 mg. of indole-propionic acid per gram of lanolin having to be used. As these concentrations are very much higher than those necessary for the solution method, it appears that the lanolin smear method will be less generally applicable than the solution method. Probably lanolin smears will be restricted to treatment of wounds on the parent plant (as after pruning) to hasten the formation of callus, or else as a technique in suitable cases where it is desired to treat a cutting before removal from its parent. Lanolin smears may also be useful in grafting, thus being a refinement on the use of dung. The work of Pearse (1936–7) suggests that spraying may be in some cases at least as effective as lanolin smears.

Cooper (1935), who applied a 1 : 2000 lanolin paste of indole-acetic acid to a scraped surface at the tops of cuttings, was able to induce formation of roots on leafless lemon cuttings, which do not ordinarily form roots. He described his experience as "very encouraging", and his 1936 paper gives additional results with cuttings difficult to root.

English Work with the Growth-Substances

Pearse (1936–7) has examined the effects of occasional and repeated spraying with solutions of phenyl-acetic and indole-butyric acids, having investigated the action of phenyl-acetic acid at greater length than any other worker. Epinasty of leaves of young tomato plants was clearly visible within thirty minutes of spraying with a 0·1 per cent. solution of phenyl-acetic acid, which could also cause the production of roots from the stem.

He wrote:

> "By the spray method of applying the growth-substances to the aerial parts of plants, uniform entry of the substance is secured so that no bending of the stem occurs. . . . The experiments described have shown that with the treatment given the growth-substances cause no increased growth of the plant as a whole, but exert, on the other hand, a reducing effect. With the appropriate concentration, this reduction is minimal.
>
> ". . . The treatments seem to have a very large effect on the way in which plant capital is distributed. The substances do in fact appear to control the amount of plant capital that shall go to the making up of each particular organ, without altering the total amount of plant material."

Effects of natural and synthetic growth-substances applied to cuttings of a number of shrubs and plants of horticultural importance have been described by Tincker (1936). Amongst the illustrations given in Tincker's second paper is one showing roots induced on cuttings of *Viburnum carlesii*, perhaps the most deliciously fragrant-flowered of all shrubs. It is hoped that use of the new synthetic growth-substances, by cheapening the cost of plants, will enable this species to be more widely grown.

Tincker's first paper (1936 *a*) is a review which may be commended to those interested in the history of the use of growth-substances on plants. His second paper (1936 *b*) describes his own experiments; he used synthetic phenyl-acetic, indole-acetic, and α-naphthalene-acetic acids, and an extract of urine (the active substance in which was mainly "heteroauxin"—indole-acetic acid), in the form of lanolin pastes of various strengths. He found tomato and *Pelargonium zonale* useful as test plants for the smear preparations. With some woody and "recalcitrant" species the lanolin-paste method of application was ineffective.

However, aqueous solutions of indole- and α-naphthalene-acetic acids were strikingly effective in inducing rooting to occur in cuttings of woody plants such as holly. Tincker's control cuttings, and those that had been treated with the active solutions, were planted in sand, not compost; otherwise his technique was similar to that used by the Boyce Thompson Institute workers. It appears evident that the Royal Horticultural Society, under whose auspices Tincker worked at Wisley, England, regard the new growth-substances as of considerable value, and a further report is promised.

Pearse and Garner (1937) have applied the solution-technique to accelerate root-production of soft-wood cuttings of plum and pear rootstocks; immersion of the bases for twelve hours in a solution containing 30 to 40 parts of α-naphthyl-acetic acid per million of water markedly improved rooting. With fig and blackcurrant cuttings, accelerated rooting was also obtained.

It seems convenient to mention here the work of van der Lek and Krijthe (1937) on the effects of indole-acetic acid solutions on the rooting of plum, quince, and other cuttings, if only because they used the East Malling types A and B (plum) and A, B, and C (quince). The various types of one species responded differently to a given concentration of substance or time of treatment. These workers are the only ones who have measured the actual uptake of solution (and, consequently, of growth-substance) by cuttings, and it appears from their account that the same dose can produce different results, depending upon the species and type of plant, and upon concentration of solution and time of administration. It was confirmed that, in general, a relatively high concentration over a short period of immersion is best. This paper offers several points of novelty, and is of considerable practical interest.

Warne (1937) has used sprays of indole-acetic acid and of α-naphthyl-acetic acids in 0·05 per cent. water solution to induce curling of chrysanthemum petals.

Uptake from Soil

Hitchcock and Zimmerman (1935) reported that the addition of synthetic growth substances to soil induced all the responses previously observed by them after applying the materials to the aerial parts of plants. There were differences in the extent and rapidity of response to substances applied to soil, these differences depending not only on the species of plants, but also upon the type of growth, magnitude, and environment of the plant. As before, only a few results can be quoted here. The plants were grown in 4-inch pots holding the equivalent of about 450 gm. of air-dry soil. The solutions were made by extensive dilution with water of an alcohol solution of each compound. Phenyl-acetic, phenyl-propionic, indole-acetic, -butyric, and -propionic, and naphthalene-acetic [? α] acids were tried.

Epinasty (downward bending) of some or all leaves of tomato plants from 4 to 10 or more inches high occurred after treatment of the undisturbed soil with any of the six acids except phenyl-propionic. Pronounced responses were often displayed within two hours of the application of the solution: plants about 5 inches high required only about 3 mg. of the substance in order to show the effect, but larger and older plants required 10 to 20 mg. On the whole, the indole compounds were more effective than the phenyl; and toxic effects were observed after application of more than 20 mg. of the indole compounds [to the youngest plants?] or about 50 mg. of the phenyl compounds.

The responses of tobacco plants were generally similar to those exhibited by tomatoes, except that no noticeable response was evoked in tobacco in less than three to six hours.

Root growth from the stems of tomatoes was essentially the same after application of the active substances to the soil as has already been described for local applications. However, "Roots induced by phenyl-propionic acid (applied to soil) appeared sooner and in larger numbers during the first eight days than did roots induced by other compounds. Phenyl-propionic acid was the only compound to induce many roots to appear from parts of the stem that did not exhibit noticeable swelling or other type of proliferation. Naphthalene-acetic acid was the most toxic to root systems in soil. . . . In addition to root growth from the stem of the tomato, roots also appeared from the petiole [leaf-stalk] and along the entire midrib of the middle leaves. This type of response was induced by indole-propionic acid (27 mg. per pot). . . . Indole-butyric acid was especially effective in causing roots to be initiated in stems of tobacco plants 10 to 12 inches in height." Optimum initiation of aerial roots in tobacco resulted from the addition of 27 mg. of indole-butyric acid to the soil.

These relatively heavy doses are made to seem enormous by the work of Grace (1937), who suggests that if young lettuce, tomato, and other plants growing in sand or soil are treated with a solution containing indole-acetic acid or its relatives, a real stimulation is obtained with a dose of active substance at the rate of 50 to 250 mg. *per acre* (120 to 600 mg. per hectare). This is—so far—the ultimate in agricultural microtherapy, and, should active substances be present in organic manures in only corresponding traces, their detection will provide a problem for the analyst.

Grace has also proposed two dusting techniques, which he claimed to be safer than treatment with solutions. (Some toxic effects of solutions are discussed on page 63 *seqq.*) Talc, or a standard mercurial dust disinfectant such as is used for cereals, was found to be a suitable carrier. Seeds of wheat, barley, and other plants were treated with a dust containing about 5 parts of growth-substance per million of dust, and were then grown on in sand. Stimulation of growth of aerial parts and of the growth of normal (underground) roots of wheat and barley was obtained with concentrations of this order.

Grace has also obtained excellent root-production on woody cuttings, by dipping the bases of bunches of 50 into a dust bearing 0·1 per cent. of growth substance by weight, shaking off the excess dust, and planting immediately. If this technique can be generally used, it should be very valuable.

The substances tried by Grace in his very promising study included indolyl-acetic, -propionic, -butyric, and α-naphthyl-acetic acids, and some salts and mixtures of these.

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CHAPTER V

GROWTH-SUBSTANCES FROM NATURAL SOURCES

Agricultural Researches Having a Possible Bearing on Growth-Substances in Soil and Manure

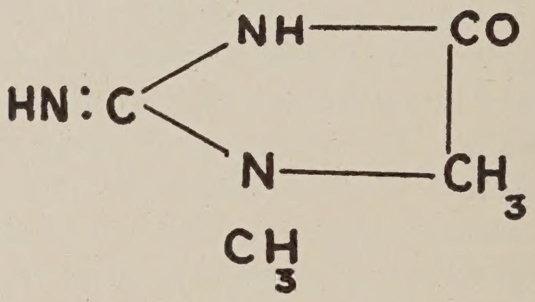
> I have experienced, that the black water taken from a Dunghill, will make a *Cabbage*, or any of that Race, prosper extremely.—R. BRADLEY, *New Improvements of Planting and Gardening*, 1718.

As already mentioned in Chap. III, the action of mixed micro-organisms on proteins and tryptophan (3-indole-α-amino-propionic acid) produces indole-acetic acid and other growth-substances, as well as substances capable of acting as nutrient materials for plants. In nature and in agricultural practice, such substances reach the plant directly from the excretions of animals or via manure-heaps, in which they are mixed with decomposition-products of straw and other vegetable materials. Natural decay of leaves and roots, and the operation of composting, may also be supposed to yield growth-substances and related bodies. Rhizopin (indole-acetic acid) has, for example, been recognized as a product of a fungus, and fungi play an important part in the decay of plant-residues.

Recent work by plant physiologists and by botanists has tended to ignore the not inconsiderable mass of work done by botanists on the assimilation of amino-acids such as tyrosine, and by chemists on the existence of organic nitrogenous compounds in soil. The early work on amino-acids was almost all directed to proving their value as nutrients (sources of nitrogen) when the amino-acid was used as sole source of nitrogen. It therefore has only a comparative interest.

On the other hand, the work of the school of Schreiner and Shorey seems to bear with some directness on the question of the relation of protein-decomposition products and soil fertility. The studies of Schreiner and Shorey on nitrogenous compounds were more concerned with bodies related to the purine group than those of the indole group and phenylic amino-acids. After their preparation of creatinine from soil, they broke new ground in two respects (Schreiner *et al.*, 1911): by trying the effect of creatinine on wheat-plants in water-cultures both with and without nitrate, and by demonstrating the presence of creatinine in stale extract of farmyard manure and in fresh cowpea vines.

Creatinine, and its relative creatine, which was also tested on wheat-plants, may have acted simply as nutrients. Creatinine is the anhydride of creatine, which is methyl-guanidino-acetic acid.



creatinine.

The experiments of Schreiner *et al.* were not fine enough for it to be possible to say that the increases of plant yield observed with creatinine and creatine in presence of small amounts of nitrate showed that creatinine, or creatine, is a growth-substance. The importance of the work of Schreiner and Shorey in the present connexion is rather that it focuses attention on the organic constituents of soils. As Schreiner and Brown wrote, in 1912, "the purely mineral-requirement theory of soil fertility has proven itself inadequate to cope with the accumulated facts."

A re-reading of the paper by Schreiner and Lathrop (1912) suggests in the light of present-day knowledge that decomposition products of protein (not necessarily of nucleo-proteins only) may be associated with the phenomena of "sickness" of highly-manured greenhouse soils, such as is treated by means of partial sterilization: growth-substances of the indole and other groups can be toxic in too great a concentration.

In a letter to *Nature* under the title "Rôle of Heteroauxones in Legume Nodule formation, Beneficial Host Effects of Nodules, and Soil Fertility", Link (1937) has suggested that "the beneficial effects of suitable concentrations of β-indole-acetic acid and other auxones may account in part for the characteristically beneficial effects of: (1) nodules for some host plants; (2) green manuring with nodule-bearing plants; (3) fertilizing with manures rich in dung and urine, or with compost; (4) humus soil; and (5) mycorrhizal fungi for some host plants."

This rather large claim has been anticipated to some extent by other workers[1], and notably at some length in relation to green manures by Schreiner, Reed, and Skinner in 1907. Whereas unchanged tyrosine was found by Schreiner, Reed and Skinner (1907) to be toxic to young wheat seedlings, a tyrosine solution, after having become discoloured on standing, produced excellent growth of young wheat seedlings. The colour of this old tyrosine solution resembled that of manure extract, and the wheat plants grown in it resembled plants grown in manure extract. The darkening was possibly due to the oxidation of tyrosine to homogentisic acid (2:5-dihydroxyphenyl-acetic acid) which gave rise to further oxidation compounds of dark colour. The effect on plants of homogentisic acid has not otherwise been investigated. The chemical relationships of this substance have received attention owing to the occurrence of homogentisic acid in the urine of people subject to the rare condition known as "alcaptonuria" (see p. 60).

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[1] In 1935 the author wrote: "One of the most remarkable effects of the leguminous crop, whether in mixture or in rotation, is its apparent ability to supplement animal manures. In peninsular Indian practice (the best-studied case) it would seem that legumes grown in mixture fill the place of animal manures. It appears unlikely that this ability is due solely to the nutrient nitrogen compounds supplied by the legumes, and it is probable that leguminous plants everywhere make a definitely biological contribution to the fertility of soil."—Hugh Nicol, "Mixed cropping in primitive agriculture," *Empire Journ. Exper. Agric.*, 1935, **3**, 189.

Another ring compound investigated by Schreiner is picoline carboxylic acid (2-methyl-4-carboxy-pyridine). This was originally isolated from a virgin Hawaiian soil by Shorey (1907) and from other soils, and appears to have afforded the first instance of isolation and identification of a definite crystalline organic compound from soil. It was isolated from the virgin Hawaiian soil to the extent of 200 to 300 parts per million of soil—a not negligible amount. It was isolated from a Maryland soil by Schreiner and Shorey (1909) by a procedure fully described, though no quantitative details were given. The Maryland soil was "exceedingly" infertile, and Schreiner and Shorey were inclined to ascribe the infertility in part to the presence of the pyridine compound. On testing its effect on wheat seedlings, these authors found that in distilled water at a concentration of about one part per million "this soil constituent . . . is a stimulant, an effect which is, however, characteristic of small doses of poisons when applied to plants." Uvitonic acid (2-methyl-4: 6-dicarboxy-pyridine) showed no clear evidence of stimulation. It seems possible that a mono-carboxy-pyridine or similar compound may yet be found to have a real "growth-promoting" effect. The Maryland soil did not give notable yield-responses to application of either farmyard manure or artificial fertilizers. Whether this failure to respond was due to sufficiency of ring-compounds, or to excess of a toxic substance such as dihydroxy-stearic acid, must remain for the present an open question.

Thornton and Smith (1914–15) found that growth of the chlorophyll-bearing unicellular animal *Euglæna* was greatly stimulated by small amounts of phenyl-alanine and tyrosine (hydroxy-phenyl-alanine; $\xrightarrow{H_2O}$ hydroxy-phenyl-acetic acid $+NH_3$), also by hay infusion; fresh hay infusion, or an infusion that has been long subject to decomposition by microbes, was appreciably less effective towards *Euglæna* than an infusion subjected for a short time to the action of microbes. Tryptophan was not used in these experiments.

Bottomley (1915–17) suggested that the quantity of auximone (growth-substance) present in stable manure increased with the progressive decomposition of the manure, though here again a very well-rotted manure contained less auximone than did fresh manure.

Marked effects of dilute extracts and fractions of farmyard manure upon the extent of production of roots (in their normal positions in water-cultures) have been reported independently by Breazeale (1927) and by Niklewski (1931, 1935).

Breazeale used several dialyzed fractions of manure on wheat seedlings and concluded that the effect of the manure extract seemed to depend largely on the presence of black organic matter, and not upon the amount of plant-food ingredients it contained.[1] Minute quantities of peat-extract had a very striking effect on the growth of citrus seedlings deprived of organic matter but well supplied with nitrogen, phosphorus, and potash. Niklewski believed that the favourable effect of very dilute extracts of manure on the root-development of plants was due to colloids, but his earlier work was not chemically critical, as he apparently performed no dialysis on his manure extracts.

In a recent publication, Niklewski and Wojciechowski (1937) have brought forward a notable dossier of evidence that minute amounts of manure and peat extracts produce marked effects on the amount of growth of several kinds of plants grown in water, sand, and soil pot, cultures. Plants supplied with the organic extracts and complete mineral nutrients grew much more sturdily than did those supplied with complete minerals only. The extracts were not dialyzed. In the discussion there is, however, but little reference to colloids.

In a few preliminary field experiments, Niklewski and Wojciechowski attempted to see whether yield effects of normal doses (25 and 50 dz. per hectare) of compost applied as a late top-dressing to cereals had a relation to the water-soluble humus substances in the compost. Increases in yield resulted, but it is not clear that these increases were significantly different from those produced by an equivalent amount of N-P-K.

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[1] Hitchcock and Zimmerman (1935, B) found that three indole acids could pass through a membrane impermeable to the dye Fast Green.

The experiments of Hartley and Greenwood (1933) which showed marked increases in crops after application of the small dressings of 1 and 2 tons of farmyard manure, were for some time believed to show a special value of farmyard manure, since the equivalent amounts of nitrogen in the form of artificials failed to produce a similar effect. It was later shown, however (Hartley, 1937) that increases similar to those given by farmyard manure in these small dressings could be obtained by the use of equivalent amounts of readily-available phosphate.

The remarkably long-lasting yield responses to farmyard manure on one of the Hoosfield (Rothamsted) classical barley plots, still await a satisfactory explanation. Plot 7-1 annually received farmyard manure at the rate of 14 tons per acre from 1852 until 1871, but has been unmanured since. The yields of both straw and grain on this plot continue to be appreciably higher than those on the unmanured plots or on those receiving certain artificials without nitrogen. The average yield (since 1871) is about the same as that of the plot annually receiving 206 lbs. of sulphate of ammonia only, but is less than that of the plots receiving 275 lbs. of nitrate of soda.

In this connexion, the rather neglected work of von Liebenberg (1916) may be recalled. For a rotation manured once with a normal dose of farmyard manure and artificials, Liebenberg showed that doses of 20 and 50 kg. of farmyard manure per hectare, applied annually each autumn thereafter, produced appreciable, though small increases, in crops. Considerable tabular details of yields and composition were given. He wrote of the 50 kg. dose that nutrients as well as organic matter were active, but in the 20 kg. dose "dürfte wohl die organische Substanz allein oder in der Hauptsache die ertragsteigernde Wirkung verursachen."

By Mr. N. W. Barritt the author has been shown quince prunings, such as are usually discarded as valueless, which had developed well-marked roots after being "skewered" during March and April, 1937, into a compost heap formed largely of grass and turves.

Root-induction and cell-elongation are distinct. Zimmerman, Hitchcock, and Wilcoxon (1936) recorded that "so far, no growth-substances have been found to accelerate elongation in root-tissue. This peculiar response of roots raises the question whether there might exist in nature an entirely different type of hormone effecting elongation of the cells." Thornton and Nicol (1936) noted that elongation of one type of cell—the root-hairs of leguminous plants—was induced by secretions of legume nodule bacteria. Some properties of these secretions have been examined, but their chemistry is still obscure. Bottomley (1915–17) found that legume nodules were a potent source of auximones.

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CHAPTER VI

SOME CONSTITUENTS OF URINE

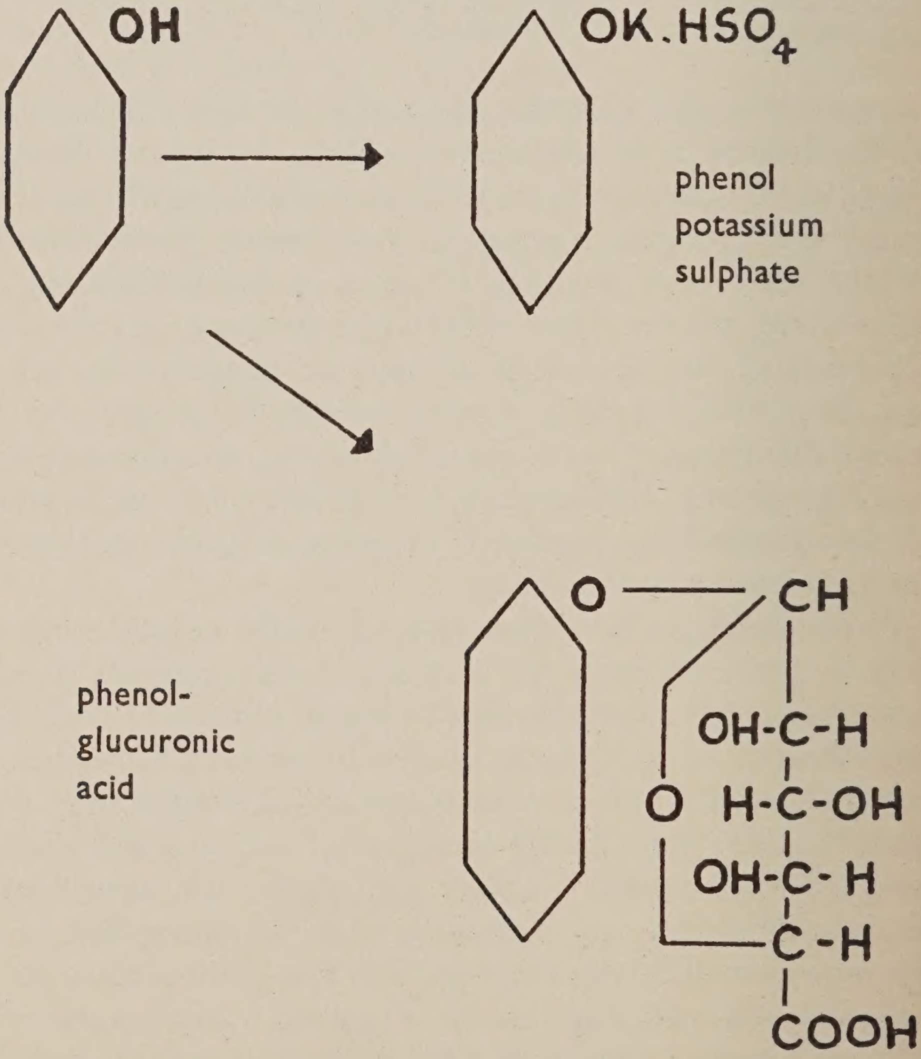
Occurrence of Growth-Substances (other than the auxins) in Urine

> Urine, in a heat below that of boiling water, yields a clear liquor, of a very disagreeable smell, and which is found to contain nothing saline.—BEAUMÉ.

A LARGE number of substances able to affect growth and differentiation in plants are to be found in urine. Some of them (auxin-a, auxin-b) probably pass unchanged through the digestive tract, and, if they do, are integral plant-products. Others are fission-products of plant or animal protein, the latter being broken down either endogenously (in the body processes of the excreting animal) or exogenously (in the digestive tract, which is outside the body proper; or, less commonly perhaps, as a result of eating decomposed food, *e.g.*, high game). Still another class is formed by those animal hormones which are capable of excretion in urine; of these the sex hormones are the chief.

In discussing urinary excretion, it should be borne in mind that to speak of urine as if it were one material, is often convenient, but does not always imply accuracy. The kind and amount of substances naturally present in urines, or excreted in urine after administration of a substance not a foodstuff, vary widely with the species, sex, age, and idiosyncrasy of the animal. Thus, phosphates are abundant in human urine, but are present in only small amounts in the urine of horned cattle; and hippuric and ornithuric acids, by their very names, remind us of special occurrences. The following outline should be read with such reservations, and with the further one that it is not intended as an introduction to urine chemistry in the wider sense.

Normally, benzene compounds possessing short side-chains are excreted as phenols. Free phenols sometimes exist in traces in urine, but phenol and cresols are usually found in urine as compounds of sulphuric acid and glucuronic acid. Such compounds are said by biochemists to be "conjugated". The combination (conjugation) prevents the phenols from attacking the walls of the urinary passage. It will be seen later that other irritant substances also have their caustic group "protected" or "detoxicated" by the formation of nearly neutral organic compounds.


OH
OK.HSO4
phenol potassium sulphate
phenol-glucuronic acid
O
CH
OH-C-H
O H-C-OH
OH-C-H
C-H
COOH

These phenolic compounds are not growth-substances. They represent the end-products of normal metabolism of such substances as tyrosine.

Phenylalanine, a constituent of almost all proteins, is oxidized in the body to phenyl-acetic acid, a growth-substance of considerable potency, and to phenyl-propionic acid. Phenyl-acetic and phenyl-propionic acids can also arise from the metabolism of tyrosine. When fed to animals, phenyl-acetic acid is said to form phenaceturic acid (a bland compound of phenyl-acetic acid and glycine) but human beings seem to be peculiar in employing glutamine as the detoxicating agent, forming phenacetoyl-glutamine.

It is interesting to note that glutamine is used to conjugate phenyl-acetic acid only, and not its derivatives. Some of the urinary phenyl-acetic acid in human urine is combined with glucuronic acid.

Phenaceturic acid (phenylacetoylglycine), being a compound of glycine with phenyl-acetic acid, is analogous to the better-known hippuric acid (benzoyl-glycine) a compound of glycine with benzoic acid. In fact the best natural source for the isolation of phenaceturic acid, as for hippuric acid, is horse urine, and both acids may be isolated from one sample of urine. It appears that the urine of herbivores is richer than that of carnivores in phenaceturic acid. The degraded sweet odour of horse urine is probably referable to its phenyl-acetic acid.

There seems to be a tendency for aromatic compounds having a fatty acid side-chain to be excreted in urine (in combination with glycine) as either benzoic or phenyl-acetic acid. The substances which, when fed, yielded benzoic acid, were those having an odd number of carbon atoms in the chain (phenyl-propionic, phenyl-valeric acids); while those acids having an even number of carbon atoms (phenyl-butyric, phenyl-caproic) appeared as phenyl-acetic acid. It will be noted that the formation of indole-acetic acid from tryptophan (indole-α-amino-propionic acid) upsets the beautiful regularity thus suggested.

The value of phenaceturic acid as a growth-regulating substance has not been tested, but it is easily hydrolyzed, and probably has the same value as its equivalent of phenyl-acetic acid. A few laboratory experiments have suggested that glycine, when used as *sole* source of nitrogen in the growing of plants, is fairly readily assimilable by plants, but such experiments bear little relation to practice. Some may be tempted to think that since phenaceturic acid comprises both a growth-substance and a source of nitrogen, it might be an especially valuable substance for plants, but it should be remembered that the amount of phenyl-acetic acid useful to the plant is probably much less than a molecular equivalent of the nitrogen required for growth. The growth-substance/nitrogen ratio in phenaceturic acid is 1, when expressed in molecules, but this is probably much too low for physiological balance; if the plant is to take up from the phenaceturic acid all the nitrogen it requires for growth, it will be left with a possibly toxic excess of phenyl-acetic acid. The point might be interesting to examine experimentally, but its nutritional implications do not seem likely to be useful. Phenaceturic acid, however, may find horticultural applications as a source of phenyl-acetic acid in cases where growth-regulation and not simple nutrition is the main object.

Hydroxy-phenyl-acetic acid in the form of *p*-hydroxy-phenaceturic acid has been reported in urine after administration of hydroxy-phenyl-ethylamine, but its importance under natural conditions is not known. It is almost certainly a growth substance. (See p. 52.)

Phenyl-propionic and β-*p*-hydroxy-phenyl-propionic acids also have been reported in urine. 2:5-dihydroxy-phenyl-acetic acid (homogentisic acid) occurs naturally in urine—in the free state, apparently, not conjugated—in persons subject to the rare condition known as "alcaptonuria". This is an inborn error of metabolism, owing to which the sufferers are unable to oxidize tyrosine and phenyl-alanine beyond the stage of the 1:2:5 compound. Alcaptonurics do not appear to suffer in health, but their urine blackens on exposure to air, owing to the rapid oxidation of the dihydroxy-benzene radical.

Chemists may wonder how tyrosine (β-*p*-hydroxy-phenyl-α-amino-propionic acid) and phenyl-alanine (β-phenyl-α-amino-propionic acid) acquire the 2:5 hydroxy groups. It may be stated that proof of the physiological origin of 2:5-dihydroxy-phenyl-acetic acid from both tyrosine and phenyl-alanine rests upon the increased excretion of homogentisic acid after ingestion of either of the amino-compounds by an alcaptonuric.

Alcaptonuria is too rare a condition to have an agricultural importance, but consideration of it suggests that the black matter of manure- and compost-heaps may be in part a complex of oxidation products of dihydroxyphenols. For further information about the acid, see the section "Nachweis and Bestimmung der Homogentisinsäure im Harn", by W. Weise, in Abderhalden's *Handbuch* (1931), Abt. 4, Teil 5 (1), 551–560.

Coupling of a ring-acid with an aliphatic amino-acid (conjugation) seems to be of a fairly general occurrence. The methyl-pyridine compounds in 2-methylpyridine and picolinic acid, after administration by mouth, appears in the urine of dogs, rabbits, and frogs, as pyridinuric acid, a compound of picolinic acid and glycine, analogous to phenaceturic and hippuric acids.

Picolinic acid administered to birds becomes combined with the peculiar avian detoxicating amino-acid, ornithine, and is excreted as pyridinornithuric acid. Ornithine is normally found in combination with benzoic acid as ornithuric acid, but acetyl-ornithine has been reported as a plant constituent by Manske.

Indoxyl (hydroxy-indole) and skatoxyl are other phenols found in urine, of carnivora particularly. They are usually conjugated in the form of potassium sulphates; the indoxyl potassium salt is known as urine-indican. Their effect on plants is not known. Indole-propionic acid is stated to undergo hydroxylation before excretion.

3-indole-acetic acid is the substance revealed by the old "urorosein" test (see Chapter IX). Its properties and a brief account of its isolation from urine are given elsewhere.

Creatine and creatinine, already mentioned on p. 50, are important and constant constituents of urine. They can almost certainly supply nitrogen to plants, at least after they have undergone bacterial decomposition. It is not known whether they can act as accessory growth-substances.

For much of the information given in this section, the author has relied upon the textbooks of biochemistry of H. H. Mitchell and T. S. Hamilton ("The Biochemistry of the Amino Acids": Chemical Catalog Co., New York, 1929, and of B. Harrow and C. P. Sherwin ("A Textbook of Biochemistry": W. B. Saunders Co., Philadelphia, 1935), and particularly in the latter the section on detoxication by A. M. Ambrose and C. P. Sherwin. See also the sections: "Nachweis und Bestimmung von Abkommlingen des Tryptophans im Harn", by W. Weise, in Abderhalden's *Handbuch*, 1931, Abt. 4, Teil. 5(1), 765–784, and "Von Tryptophan ableitbare biochemisch wichtige Verbindungen," by A. Ellinger in the same *Handbuch*, Abt. 1, Teil 7, 779–806, especially for E. and H. Salkowski's method of preparation from decomposed protein material.

To these and to similar sources the reader is referred for references and more details.

CHAPTER VII

CHEMISTRY IN RELATION TO GROWTH

Some Notions on Concentrations

Inhibition and Stimulation

THE growth-substances can act in extremely high dilutions. By the *Avena* test, auxin-a has been shown to be active at a concentration of 1 part in 110,000,000 of water; though ethylene in some circumstances is from 100 to 600 times more potent for equivalent molecular weights (Crocker, Hitchcock and Zimmerman (1935), as corrected by Hitchcock and Zimmerman (1935, B)).

Acute disturbances not unlike the effects of poisoning can be produced by the synthetic growth-substances in quite small doses; they are especially pronounced with young seedlings, grown in solution-cultures, and the question has on that account been asked whether it is not misuse of a term to call the synthetics growth-*promoting* substances (Leonian and Lilly, 1937). It is true that the substances do not always promote growth, even in large plants (Hitchcock and Zimmerman, 1935, B; Pearse, 1936–7, B) though they may initiate or control it. The doubt evoked by the toxic phenomena shown by seedlings is probably based on a misunderstanding of results of an unrealized overdosage.

This contention has received support from the work of Macht and Grumbein (1937) who have considered the question of toxicity of indole-acetic and indole-butyric acids and a naphthalene-acetic acid, from the point of view of the so-called diphasic biological effect familiar to toxicologists and pharmacologists. Their work is discussed in more detail below. In the present connexion it will suffice to quote a few words from their paper:

> "In the experiments reported by other writers (with the exception of Amlong [1936]) only the toxic phase of the drug action was recorded because of the concentrated solutions employed and the longer periods in which such plants were exposed to their action."

A fair size for the diameter of a plant cell is 0·02 mm. Supposing the cell to be a cube of 0·02 mm. side, a seedling of total volume of 0·8 ml. will have 100,000,000 cells. If all the indole-acetic acid contained in 1·0 ml. of a solution of 0·1 gm. of the acid per litre is uniformly absorbed by that seedling, each cell will contain 3·5 thousand million molecules of active substance. The number is roughly one-fiftieth of the number of molecules in a volume of air at N.T.P. equal to the volume of a cell.

Caution in dosage is still more necessary in working with excised root-tips having an initial total volume of the order of 1 cubic millimetre. As the excised tips are usually surrounded with many thousand times their weight of solution, the weight of active substance in the solution will have to be infinitesimal if harm is not to result.

Kögl *et al.* (1934) have shown that auxin-a can exert an inhibition of root-elongation in young oat seedlings. The effect was perceptible when tap water bathing the roots contained 0·01 mg. of auxin per litre. Indole-acetic acid was found to exert a similar, but less powerful, inhibition.

Marmer (1937) has made observations on the inhibition of primary root elongation in wheat seedlings, testing the three homologous acids, indole-acetic, -propionic, and -butyric, with the refinement of working at two hydrogen-ion concentrations in phosphate-buffered solutions. At *p*H 4·6 the concentrations causing a 50 per cent. reduction in root-growth were respectively 0·012, 0·250, and 0·055 mg. per litre, while at *p*H 7·5, the same results required respectively 17·58, 4·57, and 0·56 mg. per litre.

The magnitudes of such figures must depend very much upon the conditions of experiment.

The apparent paradox that synthetic growth-substances, capable of promoting growth of apical cells of seedlings, and of causing initiation of adventitious root growth, inhibit elongation of true roots, has been studied by Amlong (1936; his paper has not been seen by the author) and by Macht and Grumbein (1937). The latter workers studied a new variable—the *time* of exposure of roots (of *Lupinus albus*; white lupin) to low concentrations of indole-acetic and indole-butyric acids, and an unspecified naphthalene-acetic acid. Concentrations as low as one part of acid in 5,000,000,000 of water or salt solution (*p*H about 5) were tried. Instead of a toxic effect, a small stimulation of root-growth was observed 24 hours after transferring the plants from growth-substance solutions in which their roots had been dipped for 15-20 minutes; an exposure twice as long usually produced a small inhibition of root-growth. No estimate of error is given, however, and it is not clear whether the plants not receiving growth-substance were subjected to a control operation of transference.

Hare and Kersten (1937) have appealed to a final absence of toxicity, towards plant roots, of dilute aqueous solutions of indole-propionic acid irradiated with ultra-violet light, as evidence of the disappearance of the substance. (They give reasons for thinking that methyl anthranilate was formed).

Unfortunately, none of the published values for minimal doses of solutions affecting root-elongation is comparable with the values for minimal doses able to produce cell-elongation by the oat coleoptile test; this is true even when oats were used for determination of both types of activity. The minimum dose producing bending of the coleoptile in the usual *Avena* coleoptile test is determined from results of tests on single plants, each plant being supplied with a minute dose of substance, of which it absorbs the whole or the greater part. On the other hand, tests of the toxicity of growth-substances towards roots have been made with batches of plants having their roots bathed in relatively massive amounts of solution, and the figures for the limiting doses that inhibit root-elongation to a prescribed degree have not been furnished as a dose absorbed per plant, as must be done for comparability with the coleoptile test. In reported results of the oat coleoptile test, limiting doses have been expressed as milligrams per plant (or what is in practice the same thing, a prescribed degree of bending of so many plants per milligram or gram of growth-substance), while the limiting values for root-inhibition have been recorded as milligrams per litre of solution, with either the number of plants or the volume of solution left unspecified.

Grace (1937, B) has made an attempt to solve the paradox of growth-inhibition caused by the synthetic growth-substances. He concluded that inhibition of growth was usually due to overdosage. His colleagues Newton and Jack found that as little as 1 part of α-naphthyl-acetic acid in 200,000,000 parts of water depressed the growth of wheat roots in length, though not in weight. Grace's dust treatment of seed (see page 46) apparently gets round the paradox by slowly releasing the active agent in amounts so small as to be stimulatory.

It may be suggested here that the glycerides of acids active as growth-regulators might be safe vehicles of application. They resemble the true fats in being non-volatile and in being practically insoluble in water, but can hydrolyze slowly. Methods of preparation of some lower phenyl glycerides are given by Darzens (1937).

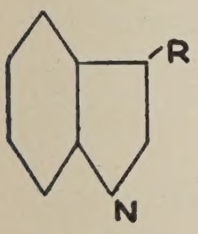
Plant-Growth Substances in Tissue Culture

Some very interesting work has been briefly reported by LaRue (1935). He cultivated minute pieces of the hypocotyl (first shoot) of dandelion, wild lettuce, and ox-eye daisy. A mineral culture solution was used, one lot receiving indole-acetic acid in the very high dilution of one part in 20,000,000. Successful growth took place only in the culture receiving the indole-acetic acid. The resulting plants developed numerous leaves and extensive root systems, and were believed by LaRue to be the first successful tissue cultures in which complete plants had been grown. See also the paper of Gautheret (1937), who found that indole-acetic acid in appropriate concentration was favourable to the growth of cambial tissue of willow.

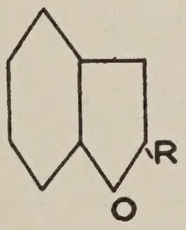
The effectiveness of salts, of esters, and of a nitrile as growth-substances shows that the favourable action of the synthetic growth-substances in proper concentration is not due to acid irritation, as has been pretended by a Continental school of plant physiologists. Quantitatively at least, the action of the natural and synthetic plant-growth substances accords with that of vitamins and hormones in animals. Very small quantities of either of the latter suffice to exert an optimal action upon the animal organism. The quantitative similarity is strengthened if the animal doses are calculated per gram instead of per kilogram of body-weight as usual. The animal is to some extent protected against overdosage of vitamins by the necessity of ingesting them through the gut. The plant itself has no obvious protection against excess of auximones offered to it in water solution, but it is possible that the colloids of manure and of soil are the plant's defence in this respect.

Chemical Constitution and Phytological Activity

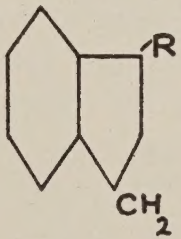
The following compounds (three of which are heterocyclic) have been shown to possess varying degrees of phytological activity, when provided with an acetic-acid side-chain. (R= —CH_2.COOH). The names under the formulæ are those of workers who have tested the action of the derivatives on plants, and are not necessarily those of the synthetizers. Thimann's coumaryl-acetic acid was called the 1-acid by him, as he excepted the oxygen atom from numbering. It is substituted in the same position as is the better-known coumarilic acid, called coumaryl-2-carboxylic acid by I eilbron (1934).[1]



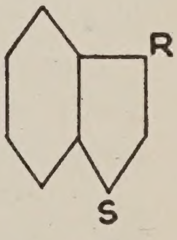
(various)



Thimann



Thimann



Shackell

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[1] The opportunity has been taken of correcting some mistakes which were made in Part III of the article in the *Manufacturing Chemist* (August 1937) before the author had access to the original of Thimann's 1935 paper.

Shackell (1937), who reported the preparation of [1]thionaphthene-3-acetic acid (by whom, is not clear, but see also the paper by Crook and Davies (1937)) found that compound inactive in the *Avena* (oat) test of coleoptile bending, but active in fairly high dilution by Went's etiolated pea method (see p. 91).

Thimann's results are most succinctly given in the form of a table, which also brings out the typical difference in activities revealed by different methods of assay, thus suggesting how much caution should be brought to attempts to correlate chemical constitution with an unspecified "activity".

TABLE.—APPROXIMATE ACTIVITY EXPRESSED AS PERCENTAGES OF THAT OF INDOLE-3-ACETIC ACID. (Thimann (1935))

	By Avena *coleoptile curvature*	*By* Avena *straight growth*	*By Went's* (1934, F) *etiolated pea method* (*p.* 91).	*By inhibition of bud-formation on etiolated pea stems.*	*By root-formation on etiolated pea cuttings Went* (1934 *B*).
Indene-3-acetic acid ...	1	7	20	14	definite[2]
Coumaryl-1 (2)-acetic acid...	0	about 0·3	4	4	definite[2]

Four other furane derivatives tested by Thimann were found to be completely inactive by the two *Avena* tests.

For synthesis, and effect on plants, of coumaryl–2(3 ?)–acetic acid, *see* V. Titoff, H. Müller, and T. Reichstein, *Helv. Chem. Acta*, 1937, **20**, 883 (from *B.C.A*).

A number of discrepant results have been reported about the "activity" or "inactivity" of substances, in relation to their effects on growth. Substances judged as "inactive" or only slightly active, by workers using one test, have been found highly active by later workers using a different test-plant and technique, and possibly evoking a different type of physiological effect on cell-growth, proliferation or differentiation.

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[1] True naphthenes are C_2H_{2n}. Thionaphthene is analogous to indene and indole, but the name thio-indene is not, however, sanctioned by custom.

[2] Dependent on mode of application, etc.

For this reason, amongst others, it has not seemed worth while to list and classify the numerous substances of which the physiological activity has been tested on plants.

A comparison of three plant tests (oat-coleoptile, etiolated pea stem, and "cylinder") has been made by Haagen-Smit and Went (1935), using a large number of synthetic substances, mostly of the indolyl and phenyl types. No naphthalene or higher aryl compound was tested in this series. *Allo*-cinnamic acid, ethyl *iso*-propyl indole-acetate, and 5-methyl-indole-acetic acid were reported to be equally as effective as 3-indole-acetic acid in the (Went's) pea test. Examined by the other two tests, no substance tried was more than a fifth as effective as indole-acetic acid, and only indole-3-pyruvic acid, 1-methyl-indole-3-acetic acid, 5-methyl-indole-acetic acid, and *allo*-cinnamic acid reached that degree of effectiveness. Though so effective in the pea and cylinder tests, *allo*-cinnamic acid and 1-methyl-indole-acetic acid were only weakly effective in the *Avena* coleoptile test, in which they produced an "abnormal" type of bending. Ordinary cinnamic acid was inactive in all three tests.

These authors wrote: "That steric conditions play an important part in the activity of these substances was confirmed by the activity of *cis-o*-methoxy-cinnamic acid, whilst the *trans* compound was inactive. In the meantime, the substitution with the methoxy groups has greatly diminished the activity." *Cis-o*-methoxy-cinnamic acid, however, was inactive in the *Avena* coleoptile test. The effects of other substitutions were briefly discussed.

The differing activities of the artificial growth-substances has been referred to their different behaviour towards what plant physiologists call "polar transport" in the plant.

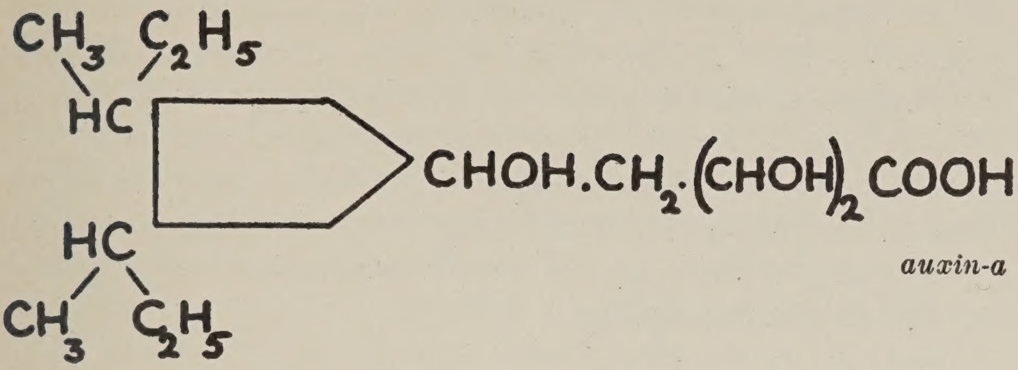
No general correlation between chemical structure and phytological activity can yet be postulated. A substance affecting growth in one species of plant may be inactive towards another species, and, even within the simple series of analogues given at the head of this section no attempt to correlate activity towards plants with the 3-substituted position can logically be made.

Tincker (1936 *a*, B) has reported that *iso*-indolinone-3-acetic acid (synthetized by Professor F. M. Rowe of Leeds) was inactive towards plants. Indole has been reported as inactive towards plants, but according to Glover (1937), the activity of skatole in the oat-coleoptile test appears to have been of the same order as that of indole-acetic acid. His suggestion that skatole may act in the plant as a growth-promoting *hormone* fails to convince, for lack of evidence that skatole is elaborated by the plant.

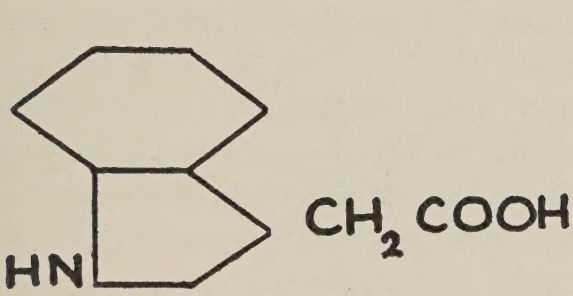
The report by Snow (1937) that benzoyl oxide and peroxide, applied in lanolin, produced perceptible bending in dark-grown oat coleoptiles, further complicates the issue. The activity of these substances was, however, only about one four-hundredth of that of indole-acetic acid.

dl-indolyl-lactic acid was the only one of several indole compounds prepared by Bauguess and Berg (1934) that was inactive as a growth-substance (Bauguess, 1935). It appears to be the only substance capable of optical activity (other than *l*-tyrosine and *l*-tryptophan) of which the growth-regulating activity has been tested, hence nothing can be said at present about the relative growth-regulating power of optical isomers. (But *see* F. Kögl, *Chem. and Ind.*, 1938, 57, 49).

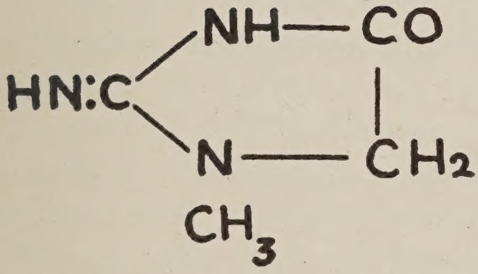
As a matter of interest, and without comment (except to point out that creatinine and creatine have not been shown to be growth substances in the modern sense of an accessory rather than a nutrient), the formulæ of auxin-a, indole-3-acetic acid, creatinine, and creatine may be given side by side:



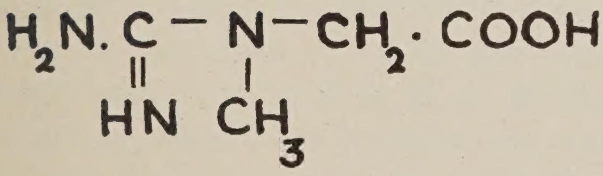
auxin-a



3-indolyl-acetic acid



creatinine



$H_2N.\underset{\underset{HN}{\|}}{C}-\underset{\underset{CH_3}{|}}{N}-CH_2\cdot COOH$

creatine

Compounds related to tyrosine as well as those related to tryptophan have physiological activity. Extracts of thyroid gland (containing thyroxin, an iodo-derivative of tyrosine) were among the first of the animal hormones shown to affect growth in plants (Nicol, 1937). Hammett (1936) writing from a zoological standpoint, has suggested that attention given to the iodine in thyroxin has overshadowed the importance of its tyrosine residue.

"*Minor*" *Elements*

A weak water solution of potassium permanganate is used by practical gardeners as a rooting medium for cuttings of some woody and semi-woody plants. Its rationale is obscure. As far as the author can gather, the permanganate is supposed to act chiefly by repressing microbial growth, such as would arise if the cuttings were left long in unchanged water. It may conceivably produce growth-substances by oxidation of tissue material of the cuttings, and it supplies potassium, which some writers overlook.

Went (1934, B) used potassium permanganate (0·05 per cent. solution in water) to give "an effective disinfection" of etiolated pea seedlings from which the roots had been cut off in the course of preparation of the seedlings for a test for "rhizocaline, the root-forming substance". It is clear that Went did not impute to potassium permanganate any positive root-forming activity on such pea seedlings, and he wrote that the salt "decreases the number of roots on the controls". Webster and Robertson (1937) have reported, in a note, that application of potassium permanganate and related salts to some (presumably intact) plants (particularly the cactus *Opuntia leucotricha* growing in sand) produced a marked growth response (? of the aerial parts), which the authors thought was not solely a manurial effect. Details are for the present lacking.

The action of some of the "minor" elements, such as boron and manganese, has been compared to that of vitamins. This is not the place for a discussion of the rôles of inorganic combination of elements necessary for plant growth, but one organic compound has a special interest as a succedaneum for inorganic iron.

Iron is usually regarded as essential for plant growth, and it is supposed to have a special part in the formation of chlorophyll, although chlorophyll contains no iron. Oddo and Pollacci (1920) found that solution-cultures deprived of iron, but supplied with other elements and with magnesium 2-pyrrole-carboxylate, permitted normal growth of several species of plants. The pyrrole ring brings to mind the indole-compounds, which, like inorganic iron, may be growth-catalysts.

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CHAPTER VIII

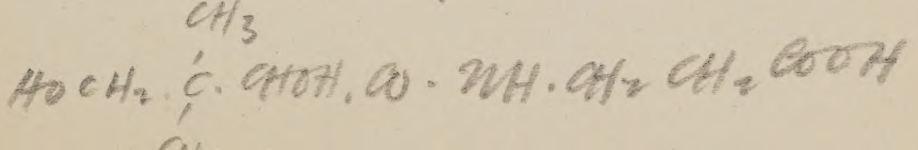
CLASSIFICATION AND NOMENCLATURE OF PLANT GROWTH-SUBSTANCES

> Becher and Stahl, pursuing further and further the combinations of the primitive principles, and of those which they term secondary, establish different orders of compound bodies, to which they give improper denominations. The signification of many of these terms is even contrary to the ideas usually affixed to them, and is liable to occasion obscurity.—BEAUMÉ.

FROM at least a historical point of view, the three following growth-substances seem to form a class: auxin-a (auxentriolic acid); auxin-b (auxenolonic acid); and "heteroauxin" (3-indole-acetic acid). To these may be added the synthetic substances, homologous with or akin to indole-acetic acid, and principally interesting the readers of this book.

In addition to these, however, there is a bewildering variety of more or less well-characterized substances derived from animal glands, for at least some of which claims have been made that they are capable of affecting plant growth (see, for example, the paper by Havas and Caldwell, 1935). Amongst these may be mentioned the male and female hormones, the chemistry of which has reached a fairly advanced stage, owing largely to the work of Marrian and of Butenandt. For the chemistry and names of most of these substances, the books by Harrow and Sherwin (1934) and Bredereck (1936) should be consulted.

Another, but ill-defined, class of substances is that known chiefly for its effects on microbial growth. Amongst these may be mentioned Williams's (1933) "pantothenic" acid, so named on account of its wide occurrence in living material,



$HOCH_2 \cdot \overset{CH_3}{\overset{|}{\underset{|}{C}}} \cdot CHOH \cdot CO \cdot NH \cdot CH_2\,CH_2\,COOH$

and Allison and Hoover's (1933) "co-enzyme R", which can be obtained from cane sugar; also reported to be widely distributed.

One more class of substances needs to be mentioned as having growth-regulating properties in plants, and that, strangely enough, is formed by the vitamins, especially vitamin C (ascorbic acid) (von Hausen, 1936).

This outline classification has been made partly for the sake of completeness, and partly to render intelligible the paragraph on nomenclature which follows: it is uneasy to discuss the action of any group of substances on plants unless we are sure which group we are discussing. No doubt the borderlines between the above-mentioned groups will shift as well as become clearer with increasing knowledge. As things are, it would be a pity to lump together in one comprehensive group such substances as: auxenolonic and ascorbic acids; the phenyl-, indolyl-, and naphthyl-acetic acids and esters and nitriles thereof; together with adrenalin and impure theelin: under some such name as "phytohormone", merely because they can all affect plant growth in some way. *Cf.* Eyster and Ellis (1924).

Group Nomenclature for Plant Growth-substances

In 1934 the author suggested the name "phytamin" as a generic term for the accessory food factors of plants (vitamins-for-plants), for which numerous names and adjectives have been proposed (auxins, plant hormones, growth-regulating and growth-promoting substances, etc.). The name ***phytamin*** was intended to cover the sense of Bottomley's (1915–17, C) "auximone", of which Bottomley wrote: "This term may usefully serve as a general descriptive name for these organic plant growth-promoting substances until our knowledge of their true nature and composition is sufficiently extended to warrant the application of a more satisfactory name." The author proposed the term "phytamin" in substitution of "auximone", because Bottomley's work was generally discredited as a result of the exaggerated claims made for his commercial preparation, "bacterized peat".

Bottomley may have been led too far in his inferences, but it can now be suggested that his laboratory demonstrations of the growth-promoting value of extracts from rotted organic matter were sound.

In view of this, it seems desirable to restore the term "auximone", which is historically valid. It should be remembered that growth-regulation and growth-promoting are not synonymous. If "auximone" is thought not to be logical (Greek *auximos*: promoting growth) there would seem to be no alternative to the generic use of either "phytamin" or growth-regulating substance: the latter may, however, be shortened to growth-substance.

The term "auxin" for the whole class of growth-substances is undesirable so long as it is also used as a trivial name for the substances at present known as auxentriolic acid (auxin-a) and auxenolonic acid (auxin-b).

What a Hormone is

The term "hormone" is inapplicable to designate a class of substances which affect the plant's growth only after having been applied from without. The first hormone was discovered by Bayliss (1924). It was a substance (not yet properly identified) secreted by a part of the intestine, and acting as a sort of release for digestive processes lower down. Previously, it was thought that body processes were controlled by telegraphic impulses along nerves, but Bayliss showed that some processes were controlled, not by intangible messages, but by actual substances. The word "hormone" was adopted to signify a chemical messenger. Whereas a painful sensation, for example, is like an unpleasant telegram, a hormone can be likened to a key, a bundle of manuscripts, or a sample of merchandise—that is, some*thing* passing between two parties and essential to the carrying out of a transaction.

Note, however, that Bayliss's hormone, and the many others discovered since, all pass between two parts of the same body. A medicine or a poison administered from without may produce a very marked result on the body, but it is not therefore a hormone. Vitamins are not hormones, because they have to be supplied to the human or animal body from a plant source—that is, from outside.

Phenyl-acetic acid or its more powerful relatives, such as indolyl-acetic acid, produce remarkable effects on plants, particularly by way of initiating adventitious root-growth. They are, however, more like vitamins than they are like animal hormones, and it would be fair to call them vitamins-for-plants. In any case, it would be misleading to continue to call them hormones, *unless* it can be proved that they are formed within the plant and act as natural chemical messengers within it.

There can be no objection to an extension of the meaning of the word "hormone" to connote substances so acting in plants. There is a small class of growth-substances, which do occur in plants and do regulate growth within them. Vitamin C is produced in the plant and regulates some growth-processes within it (von Hausen, 1936). On the basis of von Hausen's work, vitamin C is a type of true plant-hormone, in which class come also the two auxins, the plant female hormone of Butenandt, and possibly a male hormone. On the other hand, such products as those of decomposition and of putrefaction, whether derived from processes taking place in the animal gut or in the compost heap, or derived from synthetic processes, are supplied externally to the plant and cannot be called hormones: they are auximones, or vitamins-for-plants. Unless it can be proved that the "synthetic" growth-substances occur naturally in plants, and regulate growth within them, it would to-day be as absurd to give the name "hormone" to these exterior substances which effect plant growth as it would be to confuse vitamins with hormones in speaking of animal physiology.

Link *et al.* (1937) have proposed the word auxone as "a generalized term for all growth substances, including the reproductive hormones that affect growth". They say:

> "The field of growth substances is in need of tentative classification and nomenclature. Since the terms auxin and heteroauxin have been applied to specific chemical substances the collective term auxins is not satisfactory.

[So far the author can agree, remarking, however, that whilst "heteroauxin" means only 3-indole-acetic acid, "auxin" may mean either auxentriolic acid—auxin-a—or auxenolonic acid—auxin-b.] Proceeding from the Greek verb auxein, which means any increase, amplification, or waxing [*cf.* Bottomley (1915–17, C), and p. 77 of this book; it may be asked whether a verb can mean a substantive] we have used auxone as a generalized term for all growth substances including the reproductive hormones that affect growth. It includes the A and B growth substances. [What are these?] In case specific auxones should be found that induce dimensional increase (cytoauxesis), numerical increase (cytomeresis), complexity increase (cytoplasesis), or plasma increase (cytoplasmesis) of cells, they can be designated cytoauxesones, etc. Any auxone inducing more than one of these reactions may be said to be cytoauxegenic, cytomeregenic, etc. Auxones influencing reproduction may be designated genesones. Excess or deficiency in any of these processes or substances may be indicated by the prefixes hyper or hypo respectively.

"Some auxones are produced by the individual whose development they affect; these are autoauxones (auxin a and b). Others apparently do not affect growth of the plant producing them but of other plants; these are heteroauxones (heteroauxin). Heteroauxones also include growth substances not known to occur in plants (indolepropionic acid, indolebutyric acid, etc.).

"No confusion will arise if the ending 'one' is avoided in coining specific names of growth regulating substances." In the foregoing quotation, the author's interpolations are distinguished by square brackets.

"Autoauxone" is apparently an unnecessary synonym of "hormone" (*sensu stricto sive plantarum sive animalium*). A hormone is excellently characterized by the phrase of Link *et al.*: "produced by the individual whose development it affects." "Auxone" is much the same as Bottomley's "auximone", and not obviously preferable thereto. The additional term "heteroauxone" (or possibly "heterauximone") brings to the nomenclature a little more definition than does "auximone" alone, but only if "auximone" is used in the mos- general sense to mean any accessory substance (not an ordinary nutrient) affecting growth and cell-differentiation. It appears that historical and linguistic propriety would be satisfied if the term "hormone" were reserved in its usual sense, and "auximone" (or "phytamin") were used for substances affecting growth of an individual not producing them.

Link *et al.* have performed a useful service in pointing out the need for distinguishing between different modes of tissue growth, but the necessary distinction can be made without resort to ponderous words to designate the incitants of such growth-modes. It may be doubted whether the verbal polymers envisaged by Link *et al.* will come into general use. One of the simpler terms suggests, however, its own application: that Link *et al.* have been guilty of "plasesis", or complexity increase!

Chemists will not agree with the claim of Link *et al.* that "no confusion will arise if the ending 'one' is avoided in coining specific names of growth-regulating substances". If we read "trivial" for "specific", the contention may be unchallengeable. Otherwise, it seems to imply that no ketonic growth-substances must be discovered, or if they are discovered, they must not be named in accordance with the recognized rules.[1] It may be recalled that auxin-b (auxenolonic acid) possesses a ketone group.

Williams (1928) has suggested the word "nutrilite" to mean any substance that affects the growth of organisms, but the need for such a broad term does not seem to have been generally felt.

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[1] Teacher: "The guinea-pig has no tail to speak of." Small girl's recapitulatory essay: "The guinea-pig has a tail, but it mustn't be talked about."

[1] Part of this section, as well as the introductory paragraphs of Chapter I, have been reproduced from the article "What is a plant-hormone?" (*Fertiliser, Feeding Stuffs, and Farm Supplies Journal* of 28th July, 1937, **22**, 407) by kind permission of the Editor of the *Fertilizer*.

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CHAPTER IX

IDENTIFICATION OF GROWTH-SUBSTANCES AND SUBSTANCES RELATED TO THEM

> Animal substances are much more compound than those we have hitherto examined. Several of the substances furnished by animals still preserve many of the properties of the vegetables by which they are nourished.—BEAUMÉ.

THE following selected information is given in the hope that it will be useful to those proposing to undertake the search for growth-substances in agricultural materials such as manure and compost. As no work has yet been done along such lines (except for the early isolations from decomposed meat) the available information will require to be specially adapted to suit each worker's need. The existing qualitative tests refer entirely to the identification of the pure substances in aqueous solution or as they occur in urine. When urine is mentioned here, it is usually human urine that is implied.

Critical methods of identifying the growth-substances are urgently required. No progress can be made in the recognition and estimation of the substances in organic manures and decomposition products until simple yet accurate chemical tests are available. Much of the information that is available is published in medical and physiological journals difficultly accessible to agricultural and other chemists. The handbooks on organic chemistry give very little help of the kind useful for performing analysis of extracts, and it seems that the detection of ring-compounds in manure and composts will remain for some time a matter for the research laboratory able to isolate the substances for characterization by such criteria as melting points. (See Tabular Index, Chapter X). Such methods are not only laborious, but are seldom susceptible of quantitative application. Physiological tests can supply roughly quantitative information in some cases at high dilutions, but are of little critical value in distinguishing the constituents of mixtures.

Phenyl-acetic acid.—Steenbock (1912) has described a method for the estimation of phenyl-acetic acid in urine in presence of benzoic acid. He treats the urine with caustic soda with addition of hydrogen peroxide, extracts the benzoic and phenyl-acetic acids with ethyl ether, dries, and weighs after sublimation. The sublimate, of weight S grams, is titrated with $N/20$ NaOH, requiring n ml. The phenyl-acetic acid content in grams is then:

$$\frac{136\ (S - 0{\cdot}0061\ n)}{14}$$

The acid is oxidized to benzoic acid by strong oxidizing agents. It has a beautiful rose-like odour. For other tests, see the chemical handbooks.

Phenyl-propionic acid. (β-phenyl-propionic acid).—For tests see chemical handbooks. It has a pleasant, sweet odour.

Phenyl-pyruvic acid (not shown to be a growth substance) has been found in the urine of a small proportion of mental defectives by Fölling (1934). Urine containing this substance gives a deep green colour on addition of ferric chloride solution. For other properties and quantitative methods, see the paper by Penrose and Quastel (1937). Like homogentisic acid (see *infra*) phenyl-pyruvic acid is a product of incomplete metabolism; its source appears to be dietary phenyl-alanine. It is fairly easily decomposed under alkaline conditions in the presence of bacteria in urine, but the products of such decomposition are not known.

p-hydroxy-phenyl-acetic acid.—With ferric chloride solution, its solution gives a faint violet colour, quickly becoming greyish-green.

p-hydroxy-phenyl-propionic acid (β-(4-hydroxy-phenyl)-propionic acid).—Said to occur in urine. The cupric salt is difficultly soluble in water and alcohol, but is soluble in ether. (Hlasiwetz, *Journ. prakt. Chem.* (1), **72**, 401; from Beilstein's

Handbuch). The acid gives a deep red colour with Millon's reagent.

Tyrosine (*p*-hydroxy-phenyl-alanine).—Not a plant-growth substance while intact. For tests, see textbooks.

For properties and methods of isolation of some aromatic hydroxy-acids, see the sections by Waser (1923), and by P. Brigl, in Abderhalden's *Handbuch*, 1931, Abt. 4, Teil 5(1), 483–512, and Baumann's (1882) method on pp. 510–512 of the latter.

Homogentisic acid. (2: 5-dihydroxy-phenyl-acetic acid.)—The claim of this body to be considered a growth-substance is not certain, but it may be useful to give a few hints for its detection and estimation, as a guide to adoption of methods to search for it in agricultural materials. Readers who are especially interested cannot do better than consult Weise's monograph (see p. 61).

From urine containing homogentisic acid and shaken with sodium hydroxide solution and benzoyl chloride, di-benzoyl-di-hydroxyphenyl-acetamide $(C_6H_5.COO)_2.C_6H_3.CH_2.CONH_2$ separates out, which can be recrystallized from hot ethyl alcohol. M.P. 203–204°. This derivative is characteristic of alcaptonuric urine, but needs to be differentiated from tribenzamide, M.P. 201–205°, which is liable to form in small amounts when urine is benzoylated. Tribenzamide, however, is difficultly soluble in hot alcohol.

The lead salt of homogentisic acid is useful for isolation; it crystallizes in colourless or brownish prisms of M.P. 214°. It is insoluble in alcohol and ether, and only very slightly soluble in water.

With ferric chloride solution, homogentisic acid, even in fairly dilute solution, gives a transient blue colour. With Millon's reagent, an aqueous solution of the acid gives a yellow colour; on standing, a yellow precipitate develops in the cold, which turns red on heating.

A peculiar titration method has been evolved by Baumann (1891). This depends on the reduction of N/10 silver nitrate solution in urine made alkaline with ammonia. To 10 ml. of urine are added 10 ml. of 3 per cent. ammonia solution, and at once "a few" ml. of standard silver nitrate solution. The mixture is shaken, and allowed to stand for five minutes. Five drops of a 10 per cent. solution of calcium chloride and ten drops of a 20 per cent. solution of ammonium carbonate are then added, in order to form a precipitate of calcium carbonate to carry down suspended silver. The mixture is filtered, and the filtrate tested with silver nitrate. If appreciable reduction occurs, a new experiment is begun, using 2 to 5 times as much standard silver nitrate solution as first. The reduction test is repeated, and the whole thing is gone through several times, if necessary, until no reduction occurs in the filtrate. This gives an upper limit to the volume of standard silver nitrate. The experiments must again be repeated *ab initio* with successively slightly smaller doses of standard silver nitrate until no reduction occurs on the addition of silver nitrate to one portion of the filtrate, and also no or very slight cloudiness is revealed on the addition of excess hydrochloric acid to another portion. Each ml. of standard silver nitrate solution that has produced this limiting result is equivalent to 4·124 mg. of homogentisic acid. The method is probably capable of simplification.

Indole-acetic Acid. The Urorosein Test

A number of chemical reactions are available for revealing the presence of indole-acetic acid. They have been described in the literature as tests for the substance in urine, or in fairly concentrated pure solution. Some tests for the pure substance follow.

E. Salkowski's tests date from 1884 and are the best known. (1) If a 0·1 per cent. neutral solution of the acid is treated with a little very dilute ferric chloride solution, the mixture becomes cloudy; in transmitted light it appears violet, but greyish in reflected light. On careful acidification a violet-grey precipitate is formed, which gives a blue solution in ethyl alcohol.

If after adding ferric chloride and acid, more ferric chloride is added and the mixture is boiled, a cherry-red colour is produced. The colour can be removed by extraction with amyl alcohol, in which the red substance is very soluble.

(2) If to the neutral solution of the acid is added an equal volume of hydrochloric acid (D. 1·12) and a few drops of about 1 per cent. "bleaching-powder solution", a purple-red colour is produced. In concentrated solutions a precipitate is formed, soluble in ethyl alcohol and in amyl alcohol.

Ehrlich's test.—Resembling the usual diazotization reaction in hydrochloric acid solution, this is performed with alcoholic *p*-dimethylamino-benzaldehyde in hydrochloric acid in presence of nitrite. With indole-acetic acid a reddish colour is produced, not very distinct from that given by indole.

Lead acetate gives a crystalline precipitate with a 0·1 per cent. solution of sodium or potassium indole-acetate, whereas other heavy metals give little or no precipitate (Ellinger).

Indole-acetic acid is not volatile in steam, is stable towards alkalis, and is odourless.

Link *et al.* (1937, E) used the Salkowski colour test as sole chemical test for indole-acetic acid. They remark that it is considered specific for that compound. Evidence for its specificity is not quite satisfactory. An analytical scheme for the detection of the principal indole-acids and related compounds is badly needed.

Urorosein is a derivative of indole-acetic acid. It was discovered by Nencki and Sieber in 1882. Opinions differ about its constitution.

Weise in Abderhalden's *Handbuch*, Abt. 4, Teil 5(1), gives tests for the presence of indole-acetic acid (p. 767) and for the production of urorosein (p. 768) which, though similar, differ in detail. It has been suggested that urorosein is a derivative of indolyl-aceturic acid (indolyl-acetoyl-glycine), the conjugation product (see p. 58) of indole-acetic acid, but it appears uncertain that the glycine takes part in the production of urorosein.

The tests given by Weise (*loc. cit*) are as follows:

> "*Indole-acetic acid* (Salkowski's test).—If a few drops of pure nitric acid (D. 1·2) are added to a very dilute solution

> of indole-acetic acid, and then a few drops of 2 per cent. potassium nitrite solution are added, a cherry-red colour appears; if the dilution is not too high, a red colouring-matter is precipitated, which is easily soluble in ethyl acetate and in amyl alcohol. Caustic soda solution discharges the colour from ethyl acetate solution and acquires a yellow tinge. The alkaline solution is rendered colourless after addition of zinc dust and remains colourless on exposure to air. (Distinction from indole; nitroso-indole after reduction becomes blue on standing—Salkowski.)"

The same test is given by Ellinger on p. 792 of Teil 7, Abt. 1, of Abderhalden's *Handbuch.* (Urorosein may often be formed from urine by simply adding hydrochloric acid; its formation requires the presence of nitrite, which is often present in urine owing to bacterial activities). The following will now be understandable.

> "For the demonstration of urorosein, 5–10 ml. of concentrated hydrochloric acid are added to 50–100 ml. of urine. If no red colour appears after a few minutes, a little 0·2 per cent. sodium nitrite is cautiously added, drop by drop. The red colour then appears if the chromogen is present.
>
> "The dyestuff thus formed can be separated out by shaking with amyl alcohol: this operation is especially necessary when other red or yellowish-red colouring-matters are present. The amyl alcohol solution should be examined spectroscopically.
>
> "Urorosein dissolves to a reddish solution in water, ethyl alcohol, amyl alcohol, but is insoluble in ether and in chloroform (distinction from indirubin and skatole red). The colour of the solutions is discharged by addition of alkalis, and restored on acidification. The amyl alcohol solution shows a sharp absorption band in the green, about 557 Å.U."

From Weise's account of the indole-acetic acid tests with acid and nitrite, it looks as if nitroso-indolyl-acetic acid is the product. Apparently, urorosein and the indole-acetic acid nitritation compound behave differently with alkalis. On account of the apparent absence of information regarding the behaviour of urorosein in ethyl acetate, the question of the identity of the two substances in question must be left open. Plimmer (1918) wrote that urorosein is most probably nitroso-indole-acetic acid. Ellinger and Flamand (1909) and Riesser (1911) believed urorosein to belong to the group of tri-indolylmethane dyes discovered by Ellinger and Flamand; Weise (*loc. cit.*, p. 768) imputed to H. Fischer (1923) the view that it is a di-indolyl-methene derivative. Urorosein has also been studied by Herter (1908).

> Schmitz (1931), apparently quoting Riesser (1911), wrote:
>
> "Eine Darstellung des Uroroseins in Substanz ist bisher nur auf synthetischem Wege durch Oxydation von Indole-essigsäure mit Eisenchlorid oder mit Natriumnitrit und Salzsäure versucht worden."
>
> Further pursuit of the subject is left to those particularly interested.

With the modified Adamkiewicz-Hopkins (tryptophan) reaction of Winkler (1934), indole-acetic acid gives a reddish violet colour. The best ratio of reagents is 1 mol. of indole-acetic acid, 1 mol. of glyoxylic acid, 2 mol. Cu (sulphate or acetate) and excess of concentrated sulphuric acid—added in that order. (Winkler and Petersen, 1935).

For titration-curves of 3-indole-acetic, -propionic and -butyric acids in distilled water, see the paper by Albaum and Kaiser (1937).

Methods of Isolation

The *Avena* (oat) test has been used by Kögl *et al.* (1934) as a method of assay of indole-acetic acid in fractions of urine during the process of isolation. The normal urinary excretion of growth-substance was estimated by them at about 2 mg. per day, of which only about a fifth is indolyl-acetic acid, the remainder consisting chiefly of auxin-a. In the case of their special patient, "E.W.V.", who excreted 8-10 mg. of growth substance per day, at least three-quarters of that amount consisted of indole-acetic acid.

The oat assay of the successive urine fractions showed very satisfactory agreement with the determinations of indole-acetic acid by weight, allowance being made for losses and for the presence of auxin in the original urine. (The oat-test activities of the two auxins and of indole-acetic acid are very similar).

Weise (*loc. cit.*) gives the following method of preparation of indole-acetic acid from urine. It may perhaps serve as a basis of a method of isolation from agricultural materials.

Several litres of urine are evaporated down, and the residue extracted with absolute alcohol. The extract is distilled, and the residue is dissolved in a little water and acidified with sulphuric acid. It is then shaken with ether. The ether extract is separated, and is shaken with a sodium hydroxide solution to remove the indole-acetic acid. The alkaline solution is evaporated down, and the residue is repeatedly extracted with absolute alcohol to a total volume of about 100 ml. Ether is then added, and a precipitate forms. The precipitate is separated from the liquid, the latter is evaporated down, and the residue is acidified with hydrochloric acid. After an ether extraction of the acid mixture, the ether is evaporated off. This gives an impure product (containing traces of hydrochloric acid) which can be dissolved in hot benzene and recrystallized.

Kögl and others (1934, and other papers) have improved on Weise's method in several respects, notably by taking up the indole-acetic acid on an absorbent, thus avoiding the tedious evaporation. They have also made use of a chromatographic technique to separate the indole-acetic acid from auxin. Their papers should be consulted for details.

E. Salkowski's original methods of isolation of indole-acetic (1884) and indole-propionic acid (1899) from decomposed meat, on account of the presence of indole and other substances in the mixture, are too complicated to be given here, but they should be borne in mind.

Indole-propionic acid.—Ferric chloride added to a dilute solution of the acid gives a white turbidity, which becomes red on heating. Concentrated potassium nitrite and acetic acid added to a saturated solution in water form fine yellow needles of a nitroso compound, which melts at 135° with evolution of gas.

Tryptophan.—Not a growth-substance, when intact. For usual tests see textbooks.

An important modification of the well-known Adamkiewicz-Hopkins reaction has been made by Winkler (1934). This reaction depends on the blue-violet colour developed when glyoxylic acid (often present as an impurity in acetic acid) and concentrated sulphuric acid are added to free or combined tryptophan solutions. Winkler has shown that the test is inadequately given, or even fails, unless a trace of copper salt (conveniently, sulphate or acetate) is added before the sulphuric acid. The glyoxylic acid should be roughly equimolecular with the tryptophan. Under conditions described, the modified reaction is quantitative for free tryptophan at least.

Creatine and creatinine.—For properties and tests, see textbooks on human biochemistry. Creatinine was recognized by Schreiner *et al.* (1911, C) by means of its zinc chloride compound.

Oxal-acetic acid.—This is not known to be a growth-substance in the current sense, but it is assumed by Virtanen and Laine (1937) to be an intermediate in the biological fixation of nitrogen, particularly in leguminous plants. In either aqueous or alcoholic solution, both forms of the acid give an intense red colour with ferric chloride solution, wherefore it may perhaps be confused with indole-acetic acid. Oxal-acetic acid, however, has a peculiar reaction: in presence of aniline, both of its forms produce a lively evolution of carbon dioxide (Wohl, 1907), even at temperatures as low as 10°. Both forms are insoluble in chloroform and benzene (distinction from indole-acetic acid), but the higher-melting form is said to be more soluble in ether than the other is.

Went's Pea Test

A simple test for the presence of at least some growth-substances has been described by Went (1934). It was devised to test for the presence and rough quantitative estimation of auxin, but works equally well with indolyl-acetic acid.

Peas are germinated in any convenient manner (on damp cloth, sand, or blotting paper) in a darkened cupboard or room maintained at about 25° C. No nutrient solution is required, and small amounts of light do not matter so long as the shoots grow and remain straight. When the shoots are from 5 to 20 cm. long they are ready for use. The variety of peas mentioned by Went was *Alaska*.

The description that follows is given in Went's words:

> "Pieces of stem 2 to 20 cm. long are cut about 5 mm. below the terminal bud, and are used either directly or 4 to 8 hours after cutting. Immediately before application of the auxin solutions, the top of the cut piece of stem is split lengthwise by an exactly median cut with a razor blade over a length of 1 to 3 cm. If the cut is very far from median the curvatures are smaller. Upon subsequent immersion in the solution the two halves will bend out because of the tissue tension. If no auxin is present in the solution these original outward curvatures do not change any more and are fixed, since they do not go back upon plasmolysis. In the presence of auxin, however, about an hour after immersion (at 25°) the free ends of the two halves will start to bend inwards; the higher the concentration the further they bend. After about 6 hours the final stage is reached, and the originally most rapidly growing zone will be convex at the outside. If the median slit is long enough, the halves will each show an S-shape. Also, these curvatures are not changed by plasmolysis. For the appearance of the curvatures it is only necessary to have the apical cut surface in contact with the auxin-containing solutions. So either the shoots are reversed and placed upside down in a small amount of solution (5 plants together in 0·2–1 cc.) or they are left floating on the surface of the solution in

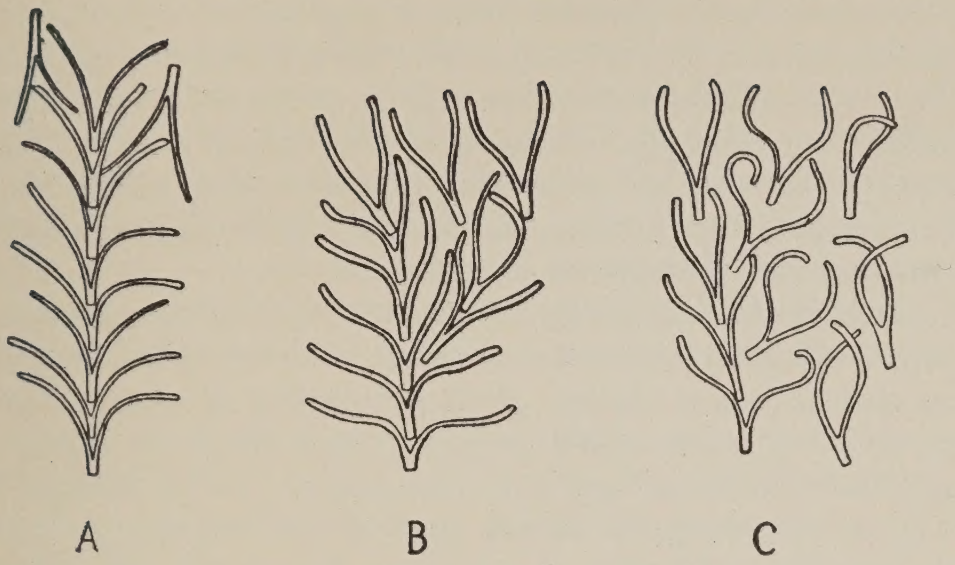
a shallow dish. If the auxin can enter only through the surface of the radial cut no curvatures appear.

"The simplest quantitative auxin determination with the pea test is to place a number of pea shoots, apically slit lengthwise over a distance of ± 10 mm. in a whole range of dilutions of the solution to be tested; 5 plants per dilution will be enough. After 12 hours it is determined in which concentration a well-recognizable reaction occurs. In this way either directly or by intrapolation the critical concentration may be calculated with an accuracy of 50 per cent."

This critical concentration, or difference of concentrations, corresponds to about 480 AE (*Avena* units), or $1{\cdot}4 \times 10^{-5}$ mg. of auxin-a, per ml. For purposes of comparison, it may be mentioned that ordinary human urine contains, per litre, about 2 mg. of auxins and indolyl-acetic acid together, each having an activity of about 50,000,000 AE per mg. Hence, Went's test is sensitive to the amount of growth substance present in about one-hundredth of a millilitre of urine. Went says: "The only work involved in this method is the making up of the dilutions and the splitting of the peas; the estimation of the reaction is done at a glance."

The curvatures can also be classified. Went's scheme is:

Degree of reaction	*Appearance of Split Stems.*
0	No auxin reaction, both halves concave at the outside over their whole length. (as A, Fig. 5.)
1	Slight auxin reaction, trace of convexity in limited regions.
2	Definite auxin reaction, ends of both halves approximately parallel.
3	Fair auxin reaction, ends bent inwards. (as B, Fig. 5.)
4	Strong auxin reaction, ends just touching.
5	Very strong auxin reaction, both halves crossing at end.
6	Very strong auxin reaction, halves crossing midway or at base (as C, Fig. 6).



(Fig. 6. (After F. W. Went, (1934)). Curvatures in split sections of pea stems, immersed in:
A, water.
B and *C*, auxin-containing solutions producing curvatures of degrees 3 (fair) and 6 (very strong) respectively.
With acknowledgments to Dr. F. W. Went and to the Editors of the "Proceedings Kon. Akad. Wet.," Amsterdam.

The crux of the preparation lies in the apparently simple recommendation to germinate the peas on a damp material. Germination is easy to accomplish, but peas are very prone to invasion by micro-organisms, often borne on the seeds themselves, and unless suitable treatment is applied, the experimenter is liable to the disappointment of finding the seedlings as a pulpy, stinking, mass. It is hoped that the following suggestions will anticipate for once the reproach that text-books give all the details except the essential ones.

The pea seeds (*Alaska*) should be externally sterilized. This may be accomplished by shaking the peas in any easily-removable disinfectant, such as free chlorine, or by washing in 95 per cent. alcohol followed by 0·2 per cent. aqueous mercuric chloride solution (not in reverse order!). A simple and effective method is to cover the peas with *concentrated* sulphuric acid (pure oil of vitriol), and leave them in it for five minutes with occasional shaking. A conical flask of large capacity is best. Whatever method of sterilization is adopted, rinsing should be performed with several changes of water. Tap water will do, as absolute sterility is not necessary. If the sulphuric acid method is used, the first addition of water to the peas should be made rapidly and in great excess, with avoidance of local overheating (for the sake of the peas), and of spurting (for the sake of the operator).

Sand is perhaps more conveniently freed from unwanted organisms than are cloth and paper, which are best sterilized by autoclaving or baking. Heat-sterilization of large amounts of sand is a tedious process of uncertain effect. A sufficient degree of freedom from micro-organisms can be attained by the use of strong chemicals, such as are available to many workers not possessing an autoclave. Here again, any reasonable method can be adopted. The following technique gives satisfactory results:

Sand *q.s.* is placed in a stout-walled shallow vessel. Better than the usual laboratory ware, a glazed "china" pie-dish fills all requirements. The sand is treated with a hot solution of potassium permanganate acidified with sulphuric acid (5 or more grams per litre of each substance, and 60° C.) and well stirred with a rod or strong spatula; the solution is renewed if necessary. After washing out the solution until only a faint pink colour remains, a slight excess of powdered sodium bisulphite is added, and the sand is intimately mixed under warm water—most effectively by using clean hands. It is important that no brown stain should be left on the sand; securing its removal demands some care. The sand is finally washed under a slow stream of running tap water, care being taken to manipulate so that every part of the sand is washed. The washing must be intimate, and need not be prolonged: mere running water will not penetrate far. Most of the excess of water being quickly drained off, the sand is ready for use. The peas should be sparsely placed, uncovered, on the more than saturated sand, and should be sprinkled with water, and inspected twice daily for the first few days of germination.

Possibly owing to the production of a volatile growth-substance, a decided curliness tends to become manifest in crowded pea shoots, hence a ventilated cupboard or dark room is better than a closed incubator, but the temperature should be maintained at about 25° C., and if small amounts of light gain entry, the vessels should be turned frequently. Some few of the peas are almost sure to turn mouldy; as soon as their condition is noticed, these should be removed with flamed forceps, and dropped into a bath of disinfectant of low surface tension. The germination cupboard should be disinfected after each crop has been grown. Time for a crop, about ten days.

Those who are thinking of performing this pea test as a research method will not need advice about its application, but those who desire merely to make a demonstration may be grateful for the hint that salt action and other effects may interfere with the test. For example, the author has found that after floating the split fragments of pea-stem on 20 ml. of liquid in a Petri dish, there was no certain response with concentrated urine, or with stale urine at any dilution. Fresh urine gave the maximum response of which it was capable—not, of course, the maximum of Went's scale—when about 4 ml. were diluted to 20 ml. Here, however, is a dislocating snag for the unwarned non-biologist. Tap water (if not chlorinated) or water from a silver-lined or glass still should be used as diluent. Water from a copper still contains enough copper to ruin many experiments with even intact plants, and these tender pea preparations are very easily hurt. My parting word to my fellow chemist is: Remember in doing any work with plants, that you are handling a structure of wonderful complexity, and alive.

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ENVOI

I have for a long time been convinced that the proper place for a preface is the end of a book. Possibly the idea is not original, but in any case I am taking my first opportunity of translating it into practice.

A preface is a message from the author to the reader. In it the author tries to nobble the reader and to tell him in a few words the aim of the book, the class of person for which it is intended, and so on. A small book like this would fail in purpose if all that sort of thing could not be grasped after a few glances at least. I would rather let the careful reader form his or her own opinions, than attempt to prejudice him or her by a preliminary note.

As to author's afterthoughts and the more or less sincere apologies that often find place in a preface, I have little to say. *Qui s'excuse, s'accuse.* Only one point should be mentioned that cannot be fairly introduced elsewhere. It is that but little reliance has been placed on abstracts and references at second-hand except as regards some analytical data; most of the sources quoted have been seen in the original by me.

Another function of a preface is to be the expression of the author's acknowledgments, a sort of grace before meat: often scamped by the reader anxious to get to the meal—though, to be sure, it is the author's grace and not the reader's. I think that the following acknowledgments will be better noted because they come at the end:

To Dr. George T. Moore, Director of the Missouri Botanical Garden, St. Louis, Missouri, U.S.A., for kind permission to reproduce Fig. 1 from the "Bulletin" of the Garden.

To Dr. A. E. Hitchcock, of the Boyce Thompson Institute for Plant Research, Yonkers, New York, U.S.A., for kind permission to reproduce Figs. 2, 3, 4 and 5 from the "Contributions" of the Institute.

To Dr. T. Eden, of the Tea Research Institute of Ceylon, for having suggested the term "growth-regulating" substance.

To some of my senior colleagues of Rothamsted Experimental Station, for having read and criticized portions of the manuscript (the blame for any mistakes remaining mine, of course).

Finally to Miss Blanche Rolt for the acuity she has applied to the deciphering of the manuscript drafts, and for the intelligent rendering which she has always given (though quite untrained in chemistry) in typing the involved technicalities.

CHAPTER X

TABULAR INDEX

of Principal Substances Mentioned (other than intermediates) with Outline of Tests and some Physical Properties

> The late M. Geoffroy the physician was the first who thought of comprizing in a table the fundamental relations or affinities in Chemistry.—Beaumé.

This table has been arranged in alphabetical order under each main heading of Miscellaneous, Phenyl, Indolyl, Naphthyl, and Other Aryl Compounds.

Solubilities are indicated qualitatively as follows: in water (w...), ethyl alcohol (a), ethyl ether (e), acetone (t), ethyl acetate (s), benzene (b), chloroform (c), methyl alcohol (m), usual fat solvents (f)—:

Almost insoluble in cold solvent: w
Appreciably soluble in cold solvent: *w*
Appreciably soluble in hot solvent: *W*
Very soluble in hot solvent: W

Almost all the substances are solid or oily at ordinary temperatures, and only one boiling-point has been given.

* An asterisk indicates corrected melting-points.

(With acknowledgements to Beilstein's *Handbuch*, Heilbron's *Dictionary*, and other authorities.)

Miscellaneous Compounds, including Inorganic and Aliphatic Substances, those of Unknown Constitution, and some trivial names

	Effects on plants, etc. page	Chemical and other tests. page
"Adco" manure	9	—
Amino-acids (general)	21–22, 36, 49	—
Auxins (general)	5, 6, 7, 30, 31, 57, 75, 77, 78, 79	91–95
Auxin-a	5, 30, 31, 63, 64, 71	31, 89
Auxin-b	5, 30, 31, 80	31
Co-enzyme R	75	—
Creatine	50, 62	—
Creatinine	50, 62	90
Dihydroxystearic acid	52	
Ethylene	38, 63	
Heteroauxin (*syn.* indole-acetic acid, *q.v.*)	15, 16, 29, 37, 43, 75	—
Magnesium compound	73	—
Manganese compounds	72	—
Mercurial seed disinfectants	46	—
Ornithine and compounds	57, 61	—
Oxalacetic acids	90	90
Pantothenic acid (composition unknown) now known '44	75	—
Phenol(s)	58	—
Picoline compounds	52, 61	—
Potassium permanganate	72	—
Pyridine compounds (*see* picoline)		
Rhizocaline (composition unknown)	36	36
Rhizopin (*syn.* indole-acetic acid, *q.v.*)	49	—
Talc	46	—
Urorosein (*see also* indole-acetic acid)	—	15–16, 85–88
Uvitonic acid	52	—
Vitamin C	75, 78	—

Phenyl Compounds

$(R{=}C_6H_5)$

A=acid; AA=acids

	Preparation, etc. page	Effects on plants, etc. page	Solubilities	M.P., °C
Benzoyl-glycine $R.CO.NH.CH_2.COOH$ (hippuric A)	57		A;W	187
– oxide $(R.CO)_2O$	—	70	*f*	—
– peroxide R.CO.O.O.CO.R	—	70	*f*	—
Cinnamic AA $R.CH_2:CH.COOH$				
– ordinary (*trans*)	—	69	*W*	133
— triglyceride	66	—	?*f*	111
– *allo-* (*cis*)	16		—	68
—*cis-o*-methoxy A	—	69	—	—
—*trans-o*-methoxy A	—	69	—	—
Hippuric A (*see* benzoyl-glycine)				
Homogentisic A	60, 61	51	b; c; *w*; *a*; *e*	152–154
Hydroxy-phenyl-acetic A	52, 60	52	—	—
Hydroxy-phenyl AA (general)	60		—	—
Phenaceturic A (*see* phenyl-glycine)				
Phenol, urinary	58	59	—	—
Phenolglucuronic A	58		—	—
Phenyl-acetic A $R.CH_2.COOH$	15, 16, 59	11, 16, 29 (esters), 38, 39, 42, 43, 45	*w*; *W*	76
— glycerides	66	66	*f*	oil
–acetoyl-glutamine	59	—	—	—
—glycine (phenaceturic A)	59	60	f; *a*; A	146?
–alanine	52, 59	52	—	—
–propionic A	16, 59, 60	45	—	
— triglyceride	66	—	*f*	oil
–pyruvic A R.CHOH.COOH	83	83	—	—
Tyrosine (*l*)	24, 61	51, 72	a; e; *w*	270 decomp.
Tyrosol	24		—	—

Chemical tests for some phenyl compounds will be found on pages 83–85.

Indole, and Compounds

Indole$=C_6H_4.\ C_2H_2.\ NH$

	Preparations, page	Effects on plants, page	Chemical tests, page	Solubilities	M.P., °C
Indole	—	70	—	w; *a*; *b*; *e*	52
–*bz*-substituted cpds.	17, 25, 26	25	—	—	—
–carboxylic cpds.	24, 25, 26, 27	—	—	—	—
—esters	17	27, 69, 29, 40, 69		—	—
–2-acetic compound	24	—	—	—	—
–3-acetic A	15, 16, 17 (isomer), 20, 21, 22, 23, 24, 25, 29, 59, 61, 88, 89	6, 7, 11, 30, 36, 37, 39, 41, 42, 43, 44, 45, 46, 51, 64, 66, 69, 92	85–89	w; *a*; *c*	164–5*; 167–8*
–3-acetonitrile	29, 30	29	—	*f*	B.P. 160 (0·2 mm.)
–3-acrylic A	70 (Bauguess and Berg, 1934)		—	w; *a*; *e*; *W*	195–196
–3-alanine (*see* tryptophan)					
–3-butyric A (normal)	22	28, 38, 39 42, 45, 46, 64	88	*a*; ?*f*	123–4*
–3-ethyl alcohol (*see* tryptophol)					
–3-ethylamine	29	—	—	w; f; *a*	146 ?
— hydrochloride	—	—	—	—	251–3*
–3-lactic A (*dl*)	70	70	—	—	144–145
–3-methylacetic A	21	70 (Kögl, 1938)	—	—	—
–3-methyl-indole (*see* skatole)					
–3-propionic A (normal)	21, 22, 24, 25, 61, 89	36, 38, 39, 45, 46, 64, 65	88, 89	dil. *a*	133–4*
–3-pyruvic A	21, 22	69		—	211 indef. ?
–3-succinic A	24	24	—	*W*	198 decomp.
–3-valeric A (normal)	25	25	—	*B*	105*
Indoxyl	61	61	—	—	—
Indylene compounds	18	—	—	—	—

Indole and compounds—contd.

	Preparations, page	Effects on plants, page	Chemical tests, page	Solubilities	M.P. °C
–1 :3-diacetic A	22	24	—	a; e; *m*; *W*	242*
Iso-indolinone-3-acetic A	—	70	—	—	—
1-methyl-R-3-acetic A	23, 27	69	—	—	—
3-methyl-R-1-acetic A	24	—	—	—	—
5-methyl-R-3-acetic A	—	69	—	—	—
Neo-indole(?) and compounds	26	—	—	—	—
Skatole and cpds.	20, 26, 27, 61	61, 70	—	*a*; *f* (skatole)	95 (skatole)
Tryptophan (indole-alanine)	20, 62	—	62, 88, 90	—	—
Tryptophol	24, 26	—	—	dil. *a*; *f*	59*

NOTE.—For absorption spectra of some indole compounds, see Hare and Kersten (1937, D); Ward (1923, A; and *ibid.*, p. 907); and Ellinger, in Abderhalden's *Handbuch*, Abt. I, Teil 7, p. 805.

Naphthyl Compounds

	Preparation, page	Effects on plants, page	Solubilities	M.P., °C.
Naphthyl-acetic AA	28, 30, 40	29, 39	—	—
—α A	28	28, 38, 41, 43, 44, 45, 46, 66	sl. *W*; *a*; *b*; *e*	131
—β A	28	Now unimportant compared with α-A	sl. *W*; *a*; *c*; *s*	142
–α -acetonitrile	29	29, 38 (40)	a; *b*; *t*	79–81
–glycollic AA	28	28	—	—
–α -glyoxylic A (glyoxalic)	28	28	*W*	98
– esters	—	40	—	—

Acetic Acid Derivatives of Other Aryl Compounds

	Preparation, page	Effects on plants, page
Acenaphthene, $C_{12}H_{10}$ (ethylene-naphthalene, $C_{10}H_6 : C_2H_4$)	29	29, 39
Anthracene, $C_{14}H_{10}$	29	29, 39
Benz-furan (*see* coumarone)		
Coumarone	68	68
Fluorene, $C_{13}H_{10}$	29	29, 39
Indene, C_9H_8	—	68
Thionaphthene (thio-indene?)	68	68

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[1] It is not certain whether this entry refers to one or two authors.

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[2] Correct attribution between these is sometimes difficult to attain.

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